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# **PHOSPHO-REGULATION OF CANCER-CAUSING HUMAN PAPILLOMAVIRUS (HPV) E6 ONCOPROTEINS**

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This thesis is submitted for the degree of Doctor of Philosophy in the Faculty of  
Life Sciences of the Open University, UK



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**ABSTRACT**

Cervical cancer develops through the combined activity of the Human Papillomavirus (HPV) E6 and E7 oncoproteins. A defining characteristic of the E6 oncoproteins derived from cancer-causing HPV types is the presence of a PDZ binding motif (PBM) at the extreme carboxy terminus of the protein, which is absent from E6 proteins derived from the so-called low risk (LR) HPV types. This PDZ binding potential of the high risk (HR) HPV E6 oncoproteins is important for their ability to support the viral life cycle and to cooperate in the induction of malignancy. However, PDZ interactions can be negatively regulated by phosphorylation within the E6 PBM. In this study, I have investigated the differential regulation of diverse HR HPV E6 PBMs. Depending on the HPV type, PDZ binding activity can be regulated by phosphorylation with PKA or AKT. This in turn inhibits PDZ recognition whilst conferring direct association with 14-3-3 family members. Such regulation is highly conserved between E6 proteins derived from HPV-16, HPV-18 and HPV-58, whilst being somewhat weaker or absent from other types such as HPV-31, -33 and -51. Phosphorylation is important for maintaining the steady state levels of HPV-18 E6 and this is also can be affected by its association with 14-3-3. I also show that HPV-18 E6 phosphorylation occurs primarily during the G2/M phase of the cell cycle whereas HPV-16 E6 phosphorylation occurs during S phase. This cell cycle-dependent phosphorylation in turn regulates the levels of E6 expression and confers enhanced interaction with multiple 14-3-3 isoforms. E6 does not

degrade 14-3-3, but it alters the subcellular distribution of 14-3-3 and as a consequence, inhibits p53/14-3-3 transcriptional transactivation in an E6 PBM dependent manner. These studies reveal unexpected differences in the regulation of HPV-16 and HPV-18 E6 function and have important implications for how phosphorylation of E6 might be expected to play a role during the respective viral life cycles and tumour development.

## CHAPTER 1: INTRODUCTION

### 1.1 Cancer

An enormous amount of effort has been invested in researching and finding novel therapeutic drugs for cancers, however, the cancer prevalence and mortality due to cancers are increasing year by year. According to the GLOBOCAN report in 2008, the number of cancer cases reported was 12.7 million and among these, there were 7.6 million of deaths. In the GLOBOCAN 2012 report (Ferlay *et al*, 2013), the global cancer burden has increased to 14.1 million new cancer cases and 8.2 million people died from cancer. The major types of cancer that lead to mortality are lung, breast, colorectal, stomach, liver, and cervical. The general global trend shows that, in developing countries, the burden of cancers related to reproduction, dietary and hormonal risk factors are increasing largely due to changes in lifestyle. Furthermore, whilst the incidence of cancer appears to be the highest in developed countries, the mortality rate is the highest in the less developed countries. It was predicted that the number of cancer cases will reach 19.3 million per year by 2025 and number of deaths will increase to over 13.1 million in 2030 (Ferlay *et al*, 2010).

Cancer is a complex disease, which can arise from all tissue types that eventually exhibit abnormal features, grow uncontrollably and then metastasize. Carcinogenesis is a multi-stage process where cells undergo physiological changes, including increased responsiveness to cell survival signals, and

reduced responsiveness to growth inhibitory signals, evade apoptosis, gain ability for cells to replicate, increased angiogenesis, and eventually cells invade or metastasized (Hanahan & Weinberg, 2000). Cancer progression involves karyotypic alteration of neoplastic cells (Pitot, 1986), with several hallmarks such as mutations, genetic instability and the gaining of unlimited replication potential (Hanahan & Weinberg, 2000; Aplan, 2006). During this process, there is a sequential accumulation of mutations that activates oncogenes and disrupts tumour suppressor genes. These events, in combination with multiple cycles of clonal selection and evolution, facilitate the process of carcinogenesis.

## **1.2 Human Papillomavirus (HPV) and Cervical cancer**

Cancer-causing viral infections, such as Hepatitis B virus (HBV) or Hepatitis C (HCV) and Human papillomavirus (HPV) contribute to 20% of cancer deaths in low and middle income countries (<http://www.who.int/cancer/en/>). Cervical cancer is the fourth most common malignancy affecting women worldwide; ranked second most common cancer in developing countries and 10th in the developed countries (Ferlay *et al*, 2013). The most important causative agents of cervical cancer are HPVs, viruses that belong to the family *Papillomaviridae*, with more than 99% of all cervical cancers being associated with HPV infection (zur Hausen, 1991, 1996). Besides causing cervical cancers, HPV infection can also lead to other anogenital cancers, such as those of vulva, anus and penis. In addition, HPV infection is associated with certain head and neck cancers (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2007).

Based on the viral DNA sequences, there are over 200 different types of HPV, of which 85 genotypes are well-characterised. Among these types, the WHO classified at least 12 as being cancer-causing agents (WHO/IVB/07.05, 2007) with 3 or 4 others as potential cancer-causing agents. In general, HPVs are grouped into cutaneous or mucosal types, depending on the origin of the infected tissues. Cutaneous types are found in the skin of hands and feet whereas mucosal types affect mucosal linings (Harwood *et al.*, 1999; Gillison and Shah, 2003; Burd, 2003). Cutaneous types such as HPV-5 and -8 have been linked to the development of non-melanoma skin cancers in immunosuppressed individuals (Harwood *et al.*, 1999). This is particularly apparent in patients suffering from Epidermodysplasia verruciformis where HPV-5 and -8 have been linked to the development of squamous cell carcinoma at sun exposed sites (Lutzner *et al.*, 1984; Pfister, 1992; Bouwes Bavinck *et al.*, 2001; Wallace *et al.*, 2012). However, the vast majority of HPV-associated cancers are caused by mucosal types.

Based on the degree of association with cervical cancer and on their ability to induce tumorigenesis in infected tissues, HPVs are categorised as either high risk (HR) or low risk (LR) types. The HR HPV types are often associated with mucosal malignancies, these include HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58 and -59 (zur Hausen & de Villiers, 1994; Bouvard *et al.*, 2009; de Villiers, 2013). Among the HR HPV types, HPV-16 and -18 are the most commonly described as cancer causing agents worldwide (Munger & Howley,

2002; Fehrman & Laimins, 2003; Münger *et al*, 2004; Snijders *et al*, 2006), especially causing invasive cervical cancers (Smith *et al*, 2007; Li *et al*, 2011a). The other six most common HR HPV types are types 31, 33, 35, 45, 52 and 58 (Clifford *et al*, 2003a; Smith *et al*, 2007). Among cervical cancers, squamous cell carcinomas (SCC) are the most common followed by adenocarcinomas (ADC) (IARC, 2007), accounting for 85% and 15% of cancers, respectively (Smith *et al*, 2007). HPV-16 is more commonly associated with SCC (55%) than ADC (33%), whereas HPV-18 is more commonly found in ADC (37%) than in SCC (13%) (Clifford *et al*, 2003b; Smith *et al*, 2007). Infection with low risk (LR) HPV types, such as HPV-6, -11, 42, -43 and -44 leads to benign genital warts (Laimins, 1993).

When the virus gains entrance to the cell, it releases its viral genomic DNA, which enters the nucleus of the infected cells as an episome. The subsequent expression of the viral genome results in microscopic changes to the cell, which ultimately results in macroscopic changes to tissue architecture. In the early stages of infection, the virus modifies the normal differentiation in the lower third of epithelial tissues, which results in the formation of low-grade squamous intraepithelial lesions (LSIL) or cervical intraepithelial neoplasia grade 1 (CIN1). In this low grade lesion, the productive viral infection may progress normally or remain unchanged for several weeks to months. Lesions at this stage are more differentiated than later lesions and can be eliminated by the immune system. The average time from the initiation of HPV infection to detection of CIN1 has been estimated to be approximately 4 months, whilst



development of CIN2 or 3 was detected after approximately 14 months (Winer *et al*, 2005). Although 99% of cervical cancers are associated with HR HPVs (zur Hausen, 1991, 1996; Walboomers *et al*, 1999), the infected woman will not necessarily develop the disease. The majority of the infections do not result in cervical cancer and most infections are cleared by the host immune system within 1 to 2 years. Approximately 5% of the HPV infection leads to cervical cancers. However, because the virus does not lyse the infected cells or cause necrosis, this aids in maintaining a persistent infection by avoiding host immune responses (Stanley, 2008).

As previously mentioned, in most cases, the viral infection is likely to be resolved by the host immune system. If this does not happen or the host is immunosuppressed, the virus can remain as a persistent infection for many years, and such infected tissues can progress to severe dysplasia or high grade squamous intraepithelial lesions (HSIL) or CIN2 and CIN3. Severe dysplastic lesions may remain as HSIL or advance to invade below the basement membrane, leading to local invasion (McMurray *et al.*, 2001). In CIN2 and CIN3 lesions, the proliferative phase is more extensive than in CIN1 and the productive stage of the viral life cycle is poorly supported (Middleton *et al*, 2003). This progression to CIN3 and ultimately invasive cancer is driven by the expression of the viral oncoproteins, E6 and E7, which leads to the hyperproliferation of the lower epithelial layers (Jeon *et al*, 1995; Jeon & Lambert, 1995; von Knebel Doeberitz, 2002). These viral oncoproteins can induce the DNA damage response by activating ATM/ATR pathway, and

abrogating cell cycle checkpoints. These favor viral genome amplification, but also result in inadequate DNA repair (Moody & Laimins, 2009; Park *et al*, 2014; Wallace & Galloway, 2014). The accumulation of DNA damage and the resulting accompanying mutations all contribute to the disease progression (von Knebel Doeberitz, 2002; Pett, 2004). Furthermore, HPVs can manipulate the host immune response by reducing inflammatory cytokines, such as IL-1, IL-6, TNF- $\alpha$  and TGF- $\beta$  in the HPV-positive keratinocytes (Arany & Tying, 1996; Fichorova, 1999; Alcocer-González *et al*, 2006; Stanley, 2008), as well as decreasing levels of interferons (Arany & Tying, 1996; Ronco *et al*, 1998; Li *et al*, 1999; Rincon-Orozco *et al*, 2009).

In high grade lesions with persistent infection, the viral genome can become integrated into the host genome, resulting in upregulation of the E6 and E7 viral oncoproteins, and loss of expression of most of the other viral genes which often occurs as a result of viral integration into the host DNA (Shirasawa *et al*, 1989; Schwarz *et al*, 1985; Jeon & Lambert, 1995). Approximately 70% of metastatic cells have integrated HPV sequences, indicating that DNA integration is likely an important step for the pathogenesis of cervical cancer (McMurray *et al*, 2001). However, episomal integration is not a prerequisite for CIN2 or CIN3 to progress to cervical cancer, although there are differences between HPV-16 and HPV-18. For cervical cancers containing HPV-16, approximately 30% of cases contain solely viral episomal DNA, whereas in the remaining 70% the viral genome is integrated (Matsukura *et al*, 1989; Pett, 2004; Vinokurova *et al*, 2008). In contrast, in the case of HPV-18, the viral

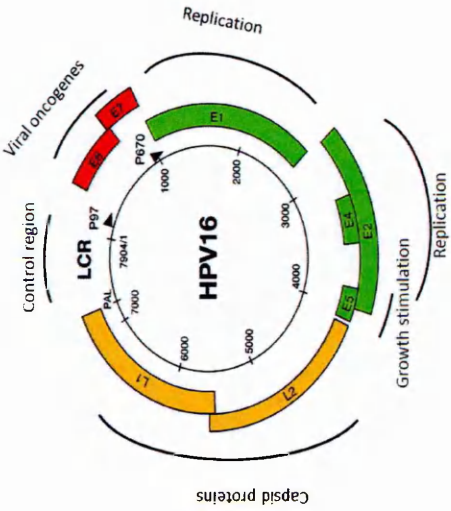
DNA is almost always integrated into the host genome in patients with HSIL and invasive carcinoma (Cullen *et al*, 1991; Pirami *et al*, 1997; Badaracco *et al*, 2002; Fehrmann & Laimins, 2003; Woodman *et al*, 2007; Vinokurova *et al*, 2008). It has been suggested that the common fragile sites (CFS) of the host chromosome, like FRA13C (13q22), FRA3B (3p14.2), and FRA17B (17q23) are the preferred site for viral genome integration, with possible normal gene expression being disrupted (Thorland *et al*, 2003). Intriguingly, the site of viral integration might be linked to the capacity of the viral transcriptional regulator, E2, to interact with Brd4 (You *et al*, 2004; Baxter *et al*, 2005), which binds E2 and the viral genome to region of interaction that is actively transcribed and is a potential viral integration site (Jang *et al*, 2014).

### 1.3 HPV genome and life cycle

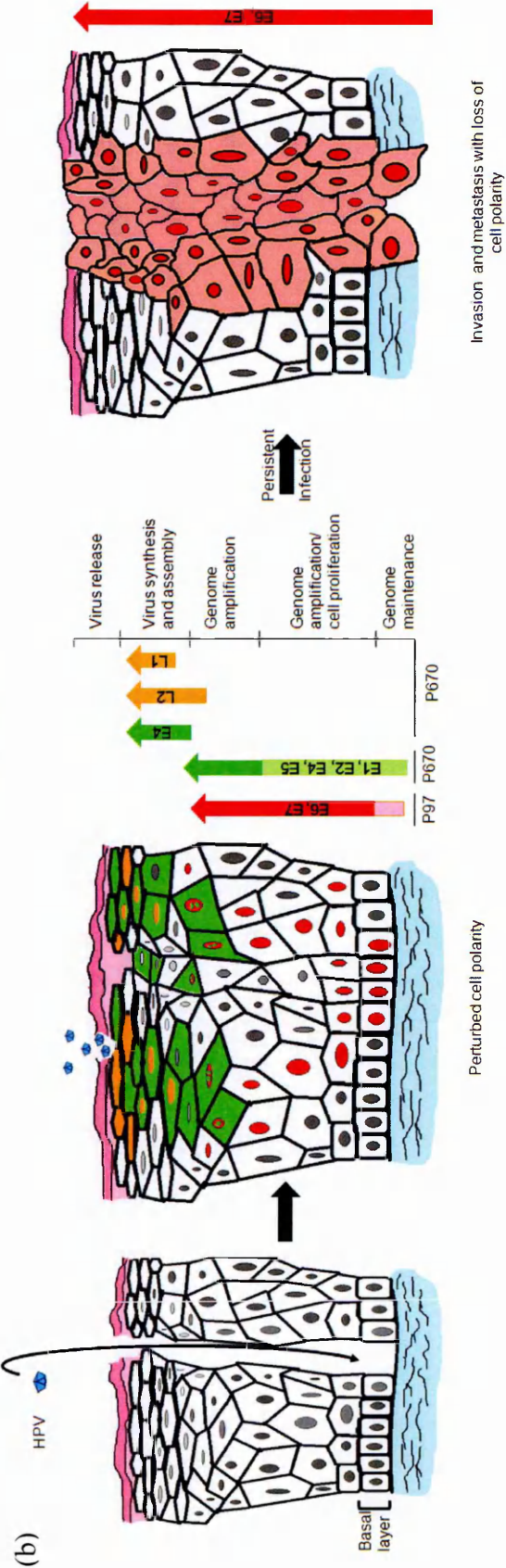
Papillomaviruses are small, non-enveloped viruses with an icosahedral capsid of approximately 50 to 60 nm in diameter, containing a circular, double-stranded DNA genome of approximately 8,000 base pairs. The genome is divided into three regions, the long control region (LCR), the early (E) region and the late (L) region and this is illustrated in Figure 1(a). The LCR is involved in regulating viral gene expression and replication. The E region, which consists of the E1, E2, E4, E5, E6 and E7 genes, encoding proteins required for viral gene expression and viral DNA replication. Many of these proteins interact with a variety of cellular target proteins, with the aim of

creating an environment favourable for the viral life cycle. The L region encodes the viral structural proteins, L1 and L2, the major and minor capsid proteins respectively.

The E1 protein is 600 to 650 amino acids in length and is the only viral protein possessing enzyme activity, being a DNA helicase. The E2 protein is 350 to 500 amino acids long and plays a pivotal role in regulating the expression of the viral genome and also enhancing viral DNA replication by recruiting the E1 protein to the viral origin of replication. E2 also has a role in ensuring the correct partitioning of viral episomes to the daughter cells during cell division by tethering the viral episomes to the host chromosome through Brd4, as mentioned above. The E4 ORF (open reading frame) is located within part of the E2 ORF, but is translated in another reading frame, it encodes a very abundant protein of 90 to 250 amino acids. E5 is a transmembrane-associated hydrophobic protein of 75 to 100 amino acids and plays a role as a minor oncoprotein. E6 is a small protein of approximately 150 amino acid residues, containing four zinc-binding Cys-X-X-Cys (CXXC) motifs, whereas E7 is a small zinc-binding protein of 100 amino acids containing two CXXC motifs. Both E6 and E7 are major oncoproteins. Both of the L1 and L2 late genes encode the viral capsid proteins of approximately 500 amino acid residues. All of these genes are coordinately expressed during the viral life cycle during the differentiation of the infected keratinocytes. The different phases of the life cycle are discussed below and is illustrated in Figure 1(b).



(a)



(b)

**Figure 1. The organisation of (a) the HPV genome and (b) viral life cycle.** (a) HPV 16 genome consists of 6 early ORFs and 2 late ORFs. The early ORF [E1, E2, E4 and E5 (in green)], as well as E6 and E7 (in red) are expressed under the influence of early (p97) and late (p670) promoters, marked by arrows, whilst the late ORFs (L1 and L2 in yellow) are expressed from p670. All viral genes are encoded on one strand of the double-stranded circular DNA genome. (b) Schematic diagram shows entrance of the viral particle into the basal cells either through a microabrasion or at the cervical transition zone. The viral genome is maintained in the lower epithelial layer as low copy number episomes. When the cells undergo differentiation, the viral genome is differentially expressed in infected cells under the p97 or p670 promoters as the cells differentiate towards the upper layer. The viral oncoproteins E6 and E7 promote S phase entry (red) in the suprabasal layer, which is necessary for viral replication. The expression of E6 and E7 in the presence of low levels of E1, E2, E4 and E5 (green) allows genome amplification and maintenance. Following this, upregulation of p670 in the upper layer of epithelial cells leads to the expression of L2 and L1 (yellow) which are required for viral synthesis and packaging. The expression of E4 in the upper layer is necessary for viral release. Upon persistent infection and lack of host immune response over years, the infected epithelial cells can develop into malignancy with loss of tissue architecture, where only E6 and E7 are expressed.

### 1.3.1 Viral Entrance

HPV infection is initiated when the viral particles gain access to basal cells through micro-trauma of the epithelium (Burchell *et al.*, 2006), or to epithelial stem cells such as those in the hair follicle (Schmitt *et al.*, 1996; Egawa, 2003). For HR HPVs, studies have shown that the epithelial cells around the squamo-columnar junction of the cervical transformation zone are more susceptible to HPV infection and cancer progression (Grayson *et al.*, 2002; Herfs *et al.*, 2012). As would be expected, the viral capsid proteins play major roles in the process of infection. The viral capsid is made up of 360 copies of L1, assembled into 72 icosohedral capsomeres, and there are believed to be a minimum of 12 copies of L2 per virus (Modis *et al.*, 2002; Buck *et al.*, 2008). L1 can self-assemble to form immunogenic virus-like particles (VLPs), even in the absence of L2 (Kirnbauer *et al.*, 1992, 1993; Hagensee *et al.*, 1993), whilst L2 is incorporated below the axial lumen of the L1 capsomeres (Buck *et al.*, 2008). The precise mechanism by which virions attach to the cell surface receptor still remains to be defined. However, it has been shown that virions first attach to the cell surface heparan sulphate proteoglycan molecules (Joyce *et al.*, 1999; Giroglou *et al.*, 2001; Shaftei-Keramat *et al.*, 2003). Following the interaction of the virions with the host cell receptors, the capsid changes its conformation, leading to exposure of the L2 amino terminus which is cleaved by secreted furin (Day *et al.*, 2008). This aids further attachment that might involve  $\alpha 6$ -integrin as the secondary receptor which is necessary for efficient HPV infection (Evander *et al.*, 1997; McMillan *et al.*, 1999; Bossis *et al.*, 2005). Following attachment, the virions are internalised via endocytosis (Selinka *et al.*, 2002;

Day *et al*, 2003; Bousarghin *et al*, 2003; Culp & Christensen, 2004). The internalised virions are subjected to intracellular trafficking which occurs through endosomal compartments via Sorting Nexin 17 (SNX17) (Bergant Marušič *et al*, 2012; Bergant & Banks, 2013). L2 interacts with SNX17 which is involved in endosomal recycling pathways, and thereby helps in avoiding lysosomal degradation (Roden *et al*, 2001; Bergant Marušič *et al*, 2012). Acidification of these compartments is important in allowing the subsequent viral uncoating and egression from endosomes (Kämper *et al*, 2006; Day *et al*, 2008; Smith *et al*, 2008; Dabydeen & Meneses, 2009). In the viral life cycle, L2 is important in transporting viral DNA through the trans-Golgi network into the host nucleus following nuclear envelope breakdown (Sun *et al*, 1995; Florin *et al*, 2004; Bossis *et al*, 2005; Pyeon *et al*, 2009; Schneider *et al*, 2011). Once in the nucleus, L2 localises to PML bodies where initiation of viral transcription is believed to occur (Sapp & Bienkowska-Haba, 2009). Meanwhile, L1 is degraded in lysosomes (Spoden *et al*, 2008).

### 1.3.2 Viral establishment

In the infected cells, the HPV genome is maintained as a nuclear plasmid, or episome, at a low copy number of approximately 10 to 200 copies. In this stage, the early viral gene products (E6, E7, E1 and E2) are expressed at a low level (Stanley *et al*, 1989; De Geest *et al*, 1993). Expression of viral DNA genomes is initiated from more than one promoter region, producing multiple viral mRNAs with several ORFs (Graham, 2010). E6 and E7 are expressed from a bicistronic mRNA (Stacey *et al*, 2000), under the influence



of the viral early promoter (p97 in HPV-16). Monolayer and raft culture indicates that AP1, the host transcription regulator, is crucial to stimulate the E6/E7 promoter activity and for the expression of both of these HPV oncogenes in the differentiating epithelial cells (Chong *et al*, 1991; Chan *et al*, 1990; Kyo *et al*, 1997; Liu *et al*, 2002). The late promoter for the viral genome is located within the E7 open reading frame. Upregulation of this promoter leads to increased expression of other viral early genes, E1, E2, E4 and E5 (Middleton *et al*, 2003). The expression of the E6 and E7 oncoproteins encourages the basal cells to proliferate into suprabasal layers (Jenkins, 2007), and the functions of these oncoproteins are discussed in a later section.

The expression of both E1 and E2 is important for maintaining viral episomal DNA (Wilson *et al*, 2002) which serves as template for viral genome amplification. E2 regulates viral DNA replication by binding to the viral LCR and recruiting the viral E1 DNA helicase to the viral origin of replication (Doorbar, 2006; Blakaj *et al*, 2009). This allows the virus to replicate its DNA which occurs independently of host cellular DNA replication during the genome establishment and amplification phases. Following initiation of viral DNA replication, E2 dissociates from the viral origin, allowing E1 to form a double hexameric ring bound to the viral DNA (Fouts *et al*, 1999; Enemark & Joshua-Tor, 2006), with its helicase functions unwinding the DNA, which then serves as template for the host cell DNA replication machinery. E1 recruits host DNA polymerase and replication protein A (RPA) to the viral origin to initiate viral DNA replication (Wilson

*et al*, 2002). E2 is a transcriptional regulator and depending on the occupancy of its recognition sites within the viral URR, it can act as either transcriptional activator or repressor. E2 also ensures that the viral episomes are retained by both daughter cells during cell division by tethering the viral DNA to the host chromosome during mitosis, through interactions with cellular proteins such as Brd4 (You *et al*, 2004; Doorbar, 2006; McBride *et al*, 2006; Parish *et al*, 2006; Blakaj *et al*, 2009).

### 1.3.3 Productive stage

From the establishment stage, the viral life cycle progresses into the productive stage which occurs when the daughter cells derived from the infected basal cells start to differentiate. This allows the amplification of the viral genomes and their ultimate packaging into new infectious viral particles. A key feature at this phase of the virus life cycle is the ability of E7 to create a S-phase like state in the infected cells. It does this by deregulating the normal cell cycle by targeting cell cycle regulatory proteins, such as the pRB family of pocket proteins (p130, pRB and p107) (Münger *et al*, 2001; Roman, 2006). These proteins interact with E2F transcription factors that regulate genes required for G1/S and G2/M transitions during the cell cycle (Classon & Dyson, 2001; Cam & Dynlacht, 2003). Interaction between E7 and pRB proteins results in the degradation of pRB, and the subsequent release of active E2F from the E2F/pRB complex (Rawls *et al*, 1990; Gonzalez *et al*, 2001; Barrow-Laing *et al*, 2010). This, in turn, promotes the expression of genes required for DNA replication and favors S-phase entry (Rawls *et al*, 1990; Tommasino & Crawford, 1995;

Gonzalez *et al*, 2001; Barrow-Laing *et al*, 2010). E7 further manipulates the cell cycle checkpoint by targeting and inactivating the cyclin-dependent kinase inhibitors (CDKIs) p21<sup>CIP1</sup> (Jones *et al*, 1997b; Funk *et al*, 1997) and p27<sup>KIP1</sup> (Zerfass-Thome *et al*, 1996). All of the above activities of E7 result in unscheduled DNA replication in the infected epithelial cells and, as a consequence, the cells mount a powerful pro-apoptotic response by upregulating the levels of p53. Thus, the E6 oncoprotein has evolved to counteract this apoptotic response by degrading p53 (Scheffner *et al*, 1990; Werness *et al*, 1990; Huibregtse *et al*, 1991; Scheffner *et al*, 1993) and Bak (Thomas & Banks, 1998) in a proteasome-dependent manner. As the cells differentiate, the viral late promoter (p670 for HPV 16) which is embedded within the E7 open reading frame is activated, resulting in expression of E1, E2, E4 and E5 genes. These viral genes encode proteins important for viral DNA replication and amplification which is independent of host cell DNA replication (Middleton *et al*, 2003). In the suprabasal layers, E1 and E2 are expressed at higher levels, and thereby increase the viral copy number (Ozbun & Meyers, 1998b).

Together, E6 and E7 create an environment favorable for viral DNA replication and amplification, this activity is also most likely augmented by the E5 protein, although there are discrepancies in the literature about its role within the viral life-cycle, depending upon the virus type and the design of the particular assay systems. E5 is a pore-forming transmembrane protein that has been described as a viroporin protein (Conrad *et al*, 1993; Wetherill *et al*, 2012). Studies have shown that E5 can modulate processing of

epidermal growth factor receptors (EGFRs), and maintains growth factor signalling and proliferation signals through promoting EGFR trafficking and dysregulation (Pim *et al*, 1992; Straight *et al*, 1993; Crusius *et al*, 1998; Adam *et al*, 2000). One consequence of this is the activation of downstream MAP Kinase signalling (Fehrmann *et al*, 2003), including activation of ERK 1/2 and p38 (Crusius *et al*, 1998, 2000). As the result of this, E1 has been proposed to accumulate in the nucleus. E5 also has potential anti-apoptotic activity through interference of FasL- and TRAIL-induced apoptosis (Conrad *et al*, 1993).

The other viral gene, E4, is primarily expressed as an E1<sup>E4</sup> fusion protein, containing 5 amino acid residues from the E1 N-terminus, as a result of mRNA alternative splicing (Chow *et al*, 1987b; Nasser *et al*, 1987; Chow *et al*, 1987a; Doorbar *et al*, 1990). E1<sup>E4</sup> is expressed most highly in cells establishing high levels of viral DNA amplification, and is considered to be a viral late protein. This is the most abundantly expressed viral protein, and although the role of E4 in the viral life cycle is the subject of much debate, numerous studies indicate that it affects G2/M progression through association with Cyclin B (Davy *et al*, 2002; Nakahara *et al*, 2002; Davy *et al*, 2002; Knight *et al*, 2011). In addition, expression of E1<sup>E4</sup> in the upper layer of the terminally differentiating epithelial cells is believed to favor viral synthesis and release by perturbing the keratin network (Doorbar *et al*, 1991; Wang *et al*, 2004; Doorbar *et al*, 1997; McIntosh *et al*, 2010).

#### 1.3.4 Viral packaging, release and re-infection

As cells continue to differentiate, the viral late genes L1 and L2 are expressed which are important for viral packaging into infectious viral particles in the upper layers of infected tissue (Ozbun & Meyers, 1998a). L2 recruits the L1 protein to PML bodies, and this is thought to enhance capsid protein packaging (Zhou *et al*, 1993; Stauffer *et al*, 1998; Florin *et al*, 2002). Prior to entering the cycle of re-infection, the virus must escape from the infected skin cells and survive the harsh extracellular environment. This is thought to be helped by E1<sup>E4</sup> protein which is present in great abundance. This forms amyloid fibres via transglutaminase 3, and helps disrupt the normal cornified envelope in the upper epithelial cells and possibly contributes to PVs survival (Wang *et al*. 2004; Brown *et al*. 2006; McIntosh *et al*. 2008). Although PVs are non-enveloped, they nonetheless are able to withstand extreme conditions, such as dehydration and high ambient temperature (Roden *et al*, 1997), and it has been shown that they can survive better when they are released from highly durable desquamated cornified cells (Bryan & Brown, 2001).

#### **1.4 Human Papillomaviruses Oncoproteins**

The HPV E6 and E7 proteins, particularly those from the high risk types, can induce malignant progression by targeting various cellular proteins involved in the regulation of apoptosis and the cell cycle, potentially causing immortalization and, eventually, cellular transformation (Mantovani & Banks, 2001; Münger *et al*, 2001). These oncoproteins target different sets

of cellular proteins, but it is the synergistic effect of both E6 and E7 that enables HPVs to disrupt the cellular homeostasis of the host.

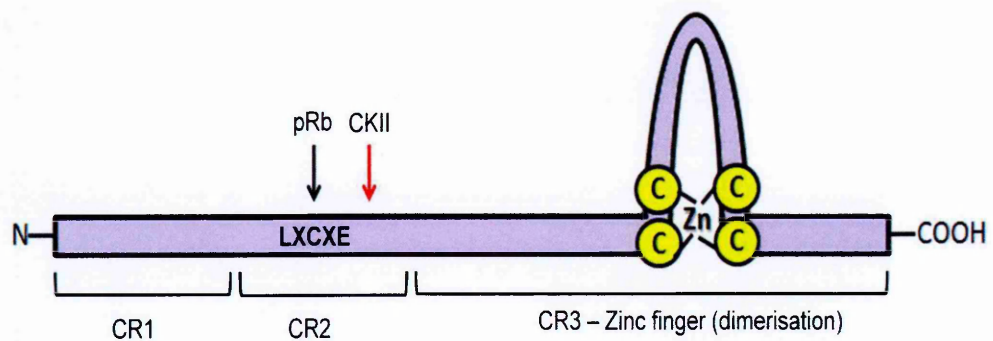
#### 1.4.1 E7 Oncoprotein

HPV E7 is a small acidic polypeptide of approximately 100 amino acids, having a molecular weight of 18 to 20 kDa; E7 was the first HPV protein to be defined as an oncoprotein, where the immortalisation and transforming capacity of E7 was identified through its ability to induce foci and colony formation in soft agar (Kanda *et al*, 1988; Phelps *et al*, 1988; Vousden *et al*, 1988; Watanabe & Yoshiike, 1988). Structurally, E7 contains 3 conserved regions (CR), CR1, CR2 at the N-terminus and CR3 at the C-terminus, as illustrated in Figure 2. It also shares a certain degree of amino acid sequence similarity with a noncontiguous sequence of the Adenovirus (Ad) E1A proteins (Phelps *et al*, 1988) within the CR2 homology domain, containing a conserved Leu-X-Cys-X-Glu (LXCXE) motif. HPV E7 is phosphorylated by Casein Kinase II (CKII) at amino acids Serine-31 (S31) and S32, which lie in close proximity to the LXCXE motif (Firzlaff *et al*, 1989; Barbosa *et al*, 1990). It is also phosphorylated at S71 by an unknown kinase in S phase of the cell cycle (Massimi & Banks, 2000). Within the CR3 domain, there are two CXXC motifs, which form a zinc-binding domain which has been shown to be the region where dimerization occurs (Barbosa *et al*, 1989; Clemens *et al*, 1995; McIntyre *et al*, 1993; Liu *et al*, 2006; Ohlenschläger *et al*, 2006). These E7 domains and interaction motifs are also shown in Figure

2. HPV E7 is localised in the cytoplasm (Smotkin & Wettstein, 1987; Huh *et al*, 2005; Nguyen *et al*, 2007; Ressler *et al*, 2007), but also can be found in the nucleus (Sato *et al*, 1989; Greenfield *et al*, 1991; Smith-McCune *et al*, 1999), and nucleolus when cells are in the G2 phase of the cell cycle (Zatsepina *et al*, 1997). E7 is a multi-functional protein. It has been reported to interact with a very large number of cellular target proteins, in many cases perturbing their normal cellular functions. Some of these interactions, with their consequences are summarised in Table 1.

E7 has a short half life of approximately 2 hrs (Smotkin & Wettstein, 1987) and is turned-over via a ubiquitin-mediated proteosomal mechanism (Reinstein *et al*, 2000) involving the cullin family of proteins. Cullin regulates the protein levels of E7 through an N-end rule, where the half life of a protein depends on the recognition of its N-terminal residues by E3 ubiquitin ligase (Varshavsky, 1996; Bachmair *et al*, 1986). E7 interacts with an E3 ubiquitin ligase complex, SCF (Skp-Cullin-F-box) for its own ubiquitination and proteolysis (Oh *et al*. 2004). On the other hand, the E7 N-terminus associates with neddylated cullin-2 (Huh *et al*, 2007) in order to induce the effective ubiquitination and degradation of pRB (Huh *et al*. 2007).

E7 interacts with retinoblastoma tumour suppressor, pRB and the related pocket proteins, p107 and p130 (Lee *et al*, 1998; Gonzalez *et al*, 2001; Helt & Galloway, 2001) through the LXCXE motif of E7, which is conserved in E1A, but is also present in HR and LR E7s (Münger *et al*, 1989; Dyson *et al*, 1989). Thus pRB binding is conserved across HPV types, regardless of their capacity to cause cancer, suggesting that interaction with pRB and



**Figure 2. Basic structure of E7 oncoproteins.** Schematic diagram shows E7 contains 3 domains: CR1 and CR2 core domain in its N-terminal part and a C-terminal CR3 or Zinc finger domain. The LXCXE motif embedded within the CR2 domain is the region important for pRB and related pocket protein targeting, and is also the region where CKII phosphorylation occurs. The C-terminal zinc finger of E7 is required for dimerisation.



**Table 1:** Summary of cellular targets of HPV E7 and manipulation of cellular events.

Cellular event manipulated	Cellular target protein	Effect of binding	Reference
Cell cycle	pRB p107 p130	Degradation; cell cycle checkpoint abrogated; Cells progress to S phase	(Lee <i>et al</i> , 1998; Gonzalez <i>et al</i> , 2001; Helt & Galloway, 2001)
	Cdk2-Cyclin A Cdk2-Cyclin E E2F-Cyclin A	Modulates cell cycle progression to S phase Activates Cdk2 kinase activity	(Dyson <i>et al</i> , 1992; Davies <i>et al</i> , 1993; He <i>et al</i> , 2003; Nguyen <i>et al</i> , 2007)
	p21 <sup>CIP1</sup> p27 <sup>KIP1</sup>	Inactivates cyclin dependent kinase inhibitors	(Jones <i>et al</i> , 1997b; Funk <i>et al</i> , 1997)
Ubiquitination	SCF (containing cullin-1/Skp)	Proteolysis of E7	(Oh <i>et al</i> , 2004a)
	Cullin-2	Ubiquitination and destabilisation of pRB	(Huh <i>et al</i> , 2007)
Cell cycle, Centrosome function and cell growth	HDAC 1 & 2	Increases transactivation and stability of Cdc25A	(Brehm <i>et al</i> , 1999; Nguyen <i>et al</i> , 2002a)
	Cdk2	Centrosome duplication	(Duensing <i>et al</i> , 2006)
	$\gamma$ -tubulin	Perturbs centrosome homeostasis	(Nguyen <i>et al</i> , 2007)
	TGF- $\beta$ 1	Blocks suppression of c-myc transcription; promote cell growth	(Pietenpol <i>et al</i> , 1990)
	Nuclear mitotic apparatus protein (NuMA)	Dynein mitotic spindle delocalisation; prometaphase delay	(Nguyen <i>et al</i> , 2008; Nguyen & Munger, 2009)
Transcription machinery	Histone acetyltransferase - pCAF - p300	Inhibits transcriptional co-activation	(Avvakumov <i>et al</i> , 2003; Bernat <i>et al</i> , 2003)

	TATA-Box binding protein, TBP and TBP-transcription factor-110 (TAF-110)	Modulate transcription initiation machinery	(Mazzarelli <i>et al</i> , 1995; Massimi <i>et al</i> , 1996)
Transformation	M phase phosphoprotein 2 (MPP2)	Enhances transcription activity and transformation	(Lüscher-Firzlaff <i>et al</i> , 1999)
	c-Jun		(Antinore <i>et al</i> , 1996)
	Insulin-like growth factor binding protein-3 (IGFBP-3)	Cellular transformation and immortalisation	(Mannhardt <i>et al</i> , 2000; Santer <i>et al</i> , 2007)
Apoptosis and DNA damage respond	ATM	Activates downstream CHK2, BRCA1, NBS1	(Moody & Laimins, 2009)
	p600	Deregulates anoikis	(Huh <i>et al</i> , 2005; DeMasi <i>et al</i> , 2005, 2007)

subsequent release of E2F is a conserved feature of HPV replication. However, regions in the carboxy terminus of E7 have also been reported to influence the efficiency of E2F release, and this might be related to its transforming capacity. However, potentially more important is the capacity of HR E7 to target pRB pocket proteins (pRB, p130 and p107) for proteasome-mediated degradation. In contrast, LR types can only degrade p107 (Zhang *et al*, 2006a), and this might be a critical difference in the respective capacity of these viruses to cause cancer. Under normal circumstances, RB represses E2F transcription activity in a cyclin/Cdk-dependent manner during late G1 phase, in this way, RB plays an important role in cell cycle regulation and differentiation. HPV E7 binds to the hypophosphorylated active form of RB (Imai *et al*, 1991), and degradation of RB subsequently leads to activation of the E2F transcription factor (Dyson, 1998). Both degradation and subsequent activation of E2F can override G1 arrest induced by p16 (Mann & Jones, 1996; Giarre *et al*, 2001). Furthermore, through perturbing pRB/E2F transcription factors which act upstream of cyclin dependent kinase (Cdk), E7 is able to abrogate the cell cycle checkpoint and permit cells to progress into S phase (Demers *et al*, 1994; Wazer *et al*, 1995; Dyson, 1998). Cdk2 forms a complex with Cyclin E or Cyclin A which regulates cell cycle progression into S phase (Koff *et al*, 1992; Ekholm & Reed, 2000). Studies showed that E7 associates with Cyclin E and Cyclin A (Dyson *et al*, 1992; Arroyo *et al*, 1993; Davies *et al*, 1993), as well as with Cdk inhibitors p21 and p27 (Zerfass-Thome *et al*, 1996; Funk *et al*, 1997; Jones *et al*, 1997a). These associations lead to activation of Cdk kinase activity and subsequently stimulate host and viral

DNA synthesis (Favre *et al*, 1977; Swindle & Engler, 1998; He *et al*, 2003; Nguyen *et al*, 2007).

Cells expressing HPV oncoproteins present with an abnormal number of mitotic poles, mitotic spindle defects, errors in chromosomal segregation, and the resultant increased genomic instability (Lengauer *et al*, 1997; Duensing *et al*, 2000). A sucrose gradient analysis indicated that E7 associates with centrosomal components such as Cdk2 and  $\gamma$ -tubulin (Nguyen *et al*, 2007). Expression of E7 increases Cdk2 kinase activity, and it also induces centrosome over duplication and abnormalities (Duensing *et al*, 2006) which occurs in part through its association with the centrosomal regulator,  $\gamma$ -tubulin (Nguyen *et al*, 2007). Besides that, in HPV E7-containing mitotic cells, the dynein mitotic spindle was found to be delocalised (Nguyen *et al*, 2008). This was later found to occur through interaction of E7 with the nuclear mitotic apparatus protein 1 (NuMA), which results in prometaphase delay (Nguyen & Munger, 2009). An indirect consequence of these activities of E7 over time might be to potentiate viral DNA integration (Kesis *et al*, 1996) and obviously to contribute to chromosomal instability during tumorigenesis.

The ability of E7 to transform primary cells (Yasumoto *et al*, 1986; Bedell *et al*, 1989; Phelps *et al*, 1992) is also potentially mediated by its ability to alter cellular apoptotic pathways, in particular anoikis. This form of apoptosis occurs when cells lack appropriate cell-matrix attachment as a result of the loss of cytoskeletal organisation (Frisch & Screaton, 2001). Cells that are resistant to anoikis gain the ability to form foci and to grow in an anchorage-independent manner (Kim *et al*, 2012). This is most likely

related to the ability of E7 to interact with the retinoblastoma protein-associated factor p600, also known as UBR4 (Huh *et al*, 2005; DeMasi *et al*, 2005). In HPV E7-containing mammalian cells, the absence of p600 results in decreased anchorage-independent growth (Huh *et al*, 2005). In contrast, the interaction of E7 with p600 results in deregulation of anoikis, hence protecting floating cells from apoptosis (DeMasi *et al*, 2007).

#### 1.4.2 E6 Oncoprotein

The HPV E6 oncoprotein is a small protein of about 150 amino acids with a molecular weight of approximately 18 kDa. Structural analysis showed that E6 is composed of a conserved scaffold with highly variable interaction surfaces involved in p53 targeting and nucleic acid binding (Nominé *et al*, 2006). The N- and C-termini are assembled in an anti-parallel manner, forming a tertiary structure of E6 with 6-stranded beta sheets and 6  $\alpha$ -helices, and its 2 zinc binding sites located at the opposite edge of the molecule (Nominé *et al*, 2006). The zinc binding sites of E6 are characterized by four CXXC motifs. HR HPV E6 is detected primarily in the nucleus (Grossman *et al*, 1989; Guccione *et al*, 2002; Tao *et al*, 2003), but is also found in the cell membrane (Grossman *et al*, 1989). Like E7, E6 is a multi-functional protein which interacts with a large number of cellular proteins, allowing E6 to be involved in regulating their normal cellular functions. The consequences of E6 association with some of these proteins are summarised in Table 2.

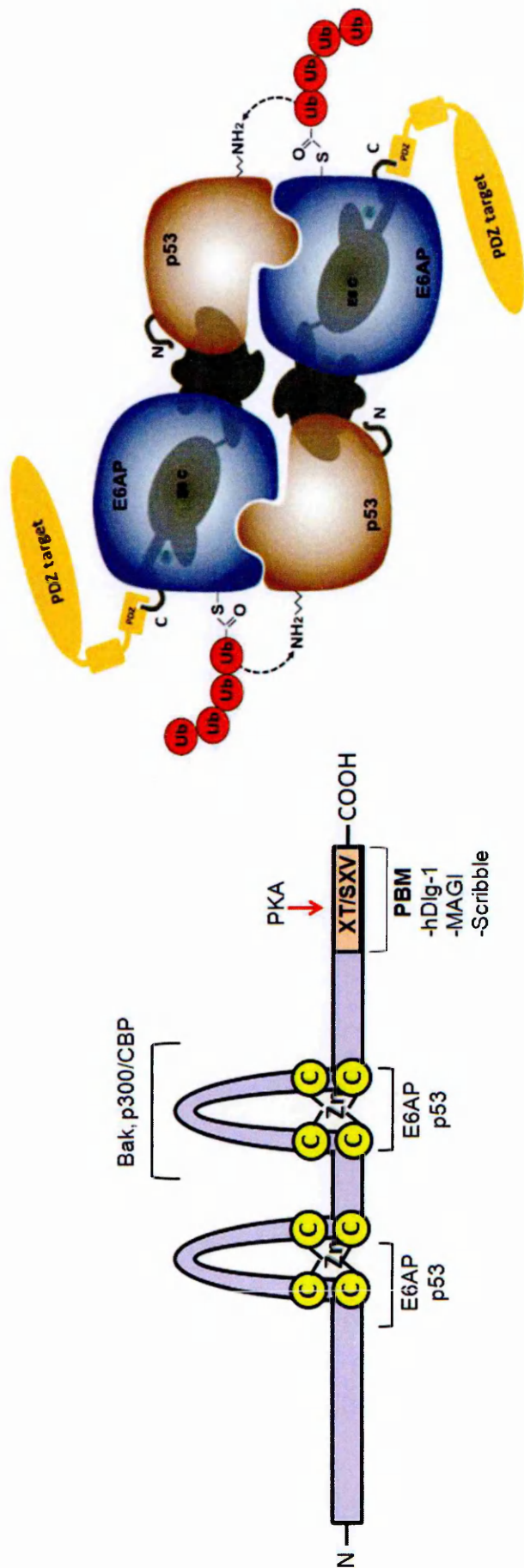
**Table 2:** Summary of HPV E6 cellular target proteins and consequence of the interaction.

Cellular event manipulated	Target proteins	Consequence of interaction	Reference
p53 and p53-related regulation	E6AP	Ubiquitination of p53 Stabilisation of E6	(Scheffner <i>et al</i> , 1993; Hengstermann <i>et al</i> , 2005)
	p53	Degradation of p53, mediated by proteasome pathway through E6AP	(Werness <i>et al</i> , 1990; Huibregtse <i>et al</i> , 1991; Scheffner <i>et al</i> , 1991, 1993)
		Loss of p53-mediated TATA-containing promoter	(Lechner <i>et al</i> , 1992)
		Abrogation of actinomycin-D-induced G1 growth arrest	(Foster <i>et al</i> , 1994)
		HPV viral genome maintenance	(Lorenz <i>et al</i> , 2013)
Apoptosis	hADA3	Degradation; loss of p53 acetylation and co-activator for p53-mediated transactivation, as well as cell senescence	(Sekaric <i>et al</i> , 2007; Shamanin <i>et al</i> , 2008)
	Bak	Degradation through E6AP	(Thomas & Banks, 1998, 1999a)
	Bcl-2	Prolonged expression	(Alfandari <i>et al</i> , 1999)
	Bax	Down-regulated by E6	
	TGF- $\beta$ 2	Downregulation of its pro-apoptotic function	(Nees <i>et al</i> , 2000)
	NF- $\kappa$ B	Activates anti-apoptotic and cell transformation through inhibition of its negative regulators -reduces levels of expression of CYLD - degrades NFX-91 - down-regulates p105	(An <i>et al</i> , 2008; Xu <i>et al</i> , 2010)
	TNF-R1 FADD Procaspase 8	Down-regulation of TNF-induced, caspase 3 and Fas-induced apoptosis	(Filippova <i>et al</i> , 2002, 2007)
Telomerase	Human	Interact and increase	(Oh <i>et al</i> ,

activity	telomerase reverse transcriptase (hTERT)	transcription activity - through Myc (E-box) and hTERT promoter - incorporation with NFX1-123 - repress p300 and Maz binding - degrades NFX <sub>1-91</sub>	2001; James <i>et al</i> , 2006; Gewin <i>et al</i> , 2004; Liu <i>et al</i> , 2009; Vliet-Gregg <i>et al</i> , 2013; Xu <i>et al</i> , 2013)
Transcription	hADA3	- SUMOylation and degradation through E6AP; abrogates activation of p53 - Abrogates cell senescence	(Sekaric <i>et al</i> , 2007; Shamanin <i>et al</i> , 2008; Chand <i>et al</i> , 2014)
	TIP60	Destabilisation; perturbs p53-dependent transcription of pro-apoptotic genes	(Jha <i>et al</i> , 2010)
	CBP/p300	Degradation leads to repression of p53 and NF- $\kappa$ B responsive genes	(Patel <i>et al</i> , 1999; Zimmermann <i>et al</i> , 1999; Thomas & Chiang, 2005)
Cell signalling	mTOR	Activation of mTOR/AKT pathway by targeting mTOR inhibitor, TSC2	(Lu <i>et al</i> , 2004a; Zheng <i>et al</i> , 2008; Spangle & Munger, 2010)
	Notch signalling and MAPK	Regulate its role in cell differentiation	(Talora <i>et al</i> , 2002b; Tan <i>et al</i> , 2012; Meyers <i>et al</i> , 2013)

E6 binds to alpha-helix containing cellular proteins with an LXXLL motif, such as the E3 ubiquitin ligase E6AP (E6-associated protein, the ubiquitin-protein ligase E3A) (Huibregtse *et al*, 1991, 1993a, 1993b) through the E/D-L/J/F-L/V-G motif in E6AP (Elston *et al*, 1998). Structural analysis reveals that the alpha helix of E6 connects both the N- and C-terminal zinc binding domains at each end of the helices to form a deep pocket which could accommodate an LXXLL peptide (Vande Pol & Klingelutz, 2013). Interaction between E6 and E6AP is crucial for E6 stability (Tomać *et al*, 2009b), and complex formation between E6-E6AP-p53 requires E6 to form a homodimer at its N-terminus (Zanier *et al*, 2012) as shown in Figure 3. Establishment of this complex is essential for the ability of E6 to direct p53 degradation which occurs through E6 association with E6AP and the subsequent recruitment of p53 where E6AP transfers ubiquitin from its C-terminal thioester cysteine bond to p53 as shown in Figure 3 (Scheffner *et al*, 1993). Overexpression of E6AP further reduces the levels of p53 and results in an increased cell population in G2 (Talis *et al*, 1998). In contrast, ablation of E6AP mimics downregulation of E6, resulting in rescue of p53 levels and restoration of p53-induced growth suppression (Hengstermann *et al*, 2005; Tomać *et al*, 2009b). Even though E6 degrades the major portion of p53 in the cells, studies suggest that the remaining pool of p53 which is not degraded by E6 remains transcriptionally active (Butz *et al*, 1995; Bañuelos *et al*, 2003; Kimple *et al*, 2013). In contrast, a recent study suggests that E6 is also able to inhibit p53-dependent transactivation upon genotoxic stress-induced DNA damage response (White *et al*, 2014). This could also occur through binding of E6 to CBP/p300 through its C terminal





**Figure 3. Basic structure of E6 oncoproteins.** The scheme on the right hand panel shows a speculative model of how E6 is believed to dimerize, thereby allowing interaction with multiple substrate partners. Note that the PBM extends beyond the central core of the E6 protein, and is believed to protrude in an unstructured conformation. Although not all targets are degraded in this fashion, such a model potentially allows interaction with more than one PDZ domain-containing substrate at any given time. The C-terminal zinc finger is involved in targeting cellular proteins, such as Bak and p300/CBP as shown on the left hand panel. Also shown is the C-terminal PDZ binding motif (PBM) which is conserved in the high risk HPV types E6s. Within the PBM, is embedded the PKA phosphorylation site; this is the region where E6 associates with PDZ containing proteins including hDlg-1, MAGI and Scribble. (Adapted from Pim *et al.*, 2012).

CXXC motif, thereby, inhibiting p53 transcriptional transactivation and the pro-apoptotic nuclear NF- $\kappa$ B transactivation activity which is modulated by p300 (Patel *et al*, 1999; Zimmermann *et al*, 1999; Thomas & Chiang, 2005). Besides p300, other histone acetyltransferases that have an effect on p53 transcriptional transactivation, such as hADA3 and TIP60, are also targeted by E6. In the case of hADA3, E6 inhibits its activity by proteasome-mediated degradation (Shamanin *et al*, 2008; Chand *et al*, 2014), which is an alternative way to inhibit p53 transcriptional transactivation (Kumar *et al*, 2002; Sekaric *et al*, 2007; Shamanin *et al*, 2008), since hADA3 functions as the co-activator for p53-mediated transactivation. Likewise, E6 can manipulate TIP60 function, where this association leads to destabilisation of TIP60 and subsequently perturbs the p53-dependent transcription of pro-apoptotic genes (Jha *et al*, 2010).

Whilst E6 can degrade p53, this however depends on the manner of the E6/p53 association. The E6 C-terminal half of all HPV types can bind to p53, but HR HPV E6 binds and degrades p53 through its N-terminus (Crook *et al*, 1991). Degradation of p53 also depends on the region of p53 associated with E6, and this appears to be different between the HR and the LR types. The HR E6 binds to the core structure of p53 for degradation mediated by E6AP. Degradation of p53 by HR HPV E6 allows cells to override actinomycin-D-induced G1 growth arrest (Foster *et al*, 1994) and to maintain the HPV viral genomes as extrachromosomal episomes (Park & Androphy, 2002; Lorenz *et al*, 2013; Brimer & Vande Pol, 2014). On the other hand, LR E6 binds to the C-terminal region of p53 (Li & Coffino, 1996). Even though LR E6 can bind p53 through E6AP, this does not result

in p53 degradation (Brimer *et al*, 2007). Instead, LR HPV E6 utilises a different approach in modulating p53, including repression of p53 transcription of TATA-containing promoters (Lechner *et al*, 1992), inhibit p53 transcription transactivation (Kiyono *et al*, 1994), retaining p53 in the cytosol (Sun *et al*, 2008) and through association with CBP/p300 even though the association is weaker than the HR HPV E6 (Patel *et al*, 1999).

Several studies suggest that for E6 proteins to exert their full oncogenic potential they require interactions with many other cellular pro- and anti-apoptotic proteins (Ishiwatari *et al*, 1994; Nakagawa *et al*, 1995). One of the first to be described is Bak, which is involved in induction of apoptosis upon a variety of stimuli, including UV irradiation. In this case, both HR and LR types of E6 bind and degrade Bak through E6AP, abrogating its pro-apoptotic function (Thomas & Banks 1998; 1999). Intriguingly, this activity also appears to be significant for the cutaneous HPV types (Jackson & Storey, 2000; Jackson *et al*, 2000), suggesting that it is an important function for many HPV types. Expression of HR HPV 16 E6 in keratinocytes enables the cells to circumvent serum- and calcium-mediated differentiation (Sherman & Schlegel, 1996; Alfandari *et al*, 1999). E6-mediated suppression of apoptosis in cells subject to serum-calcium switch occurs through manipulation of anti-apoptotic Bcl-2 and pro-apoptotic Bax. Normally, the levels of Bcl-2 decrease whilst the levels of Bax increase in serum-calcium switch cells. However, when E6 is expressed, the expression levels of Bcl-2 is increased whereas Bax levels is lower (Alfandari *et al*, 1999). E6 also modulates the Fas and caspase pathways by reducing the cellular levels of Fas-associated death domain (FADD) and procaspase 8,

respectively (Filippova *et al*, 2004, 2007). Another pro-apoptotic protein downregulated by E6 is the transforming growth factor- $\beta$ 2 (TGF- $\beta$ 2) and this also results in downregulation of the expression of TGF- $\beta$ 2 responsive genes (Nees *et al*, 2000).

Increased telomerase activity has always been implicated in cancer development. Cells with activated telomerase, an enzyme that adds TAAGGG repeats onto the telomeres (Feng *et al*, 1995), have the ability to continue to grow (Greider & Blackburn, 1985). Increment of the telomerase activity, which is normally suppressed in healthy somatic cells, is crucial for immortalisation (Kim *et al*, 1994; Artandi & DePinho, 2010). In cells containing HPV E6 and E7 oncoproteins, the length of the telomere is reduced after surviving crisis, but the length of telomere increases in late cell passages (Klingelhutz *et al*, 1994). Later studies showed that HPV E6 alone is sufficient to activate telomerase in a mechanism independent of p53 degradation (Klingelhutz *et al*, 1996) and independent of the E6-PBM (Kiyono *et al*, 1998). Many studies have shown that E6 can increase hTERT activity through multiple mechanisms. This can involve direct interactions with the enzyme itself (Liu *et al*, 2009), although in most cases it appears to be indirect through an increase in hTERT gene expression levels. This can occur through E6 interaction with Myc and upregulation of E-box dependent transcription of hTERT (Oh *et al*, 2001) or through the ability of E6 to synergize with NFX1-123 to also increase its activity (Vliet-Gregg *et al*, 2013) and downregulate the hTERT repressors, like NFX<sub>1-91</sub> (Gewin *et al*, 2004), p300 (James *et al*, 2006) and Maz which binds to the hTERT promoter (Xu *et al*, 2013).

Cellular signalling pathways play a central role in controlling and providing the link between cellular compartments. This involves activation or inhibition of cellular proteins, mainly regulated by enzymes like kinases and phosphatases. In order to be part of the cellular network, HPV E6 is involved in manipulating several cellular pathways, including the NF- $\kappa$ B-TNF, mammalian target of rapamycin (mTOR)-AKT, MAPK and Notch pathways. The mTOR-AKT, which are the key serine/threonine kinases in PI3K pathway, is activated by E6 (Sabatini, 2006; Spangle & Munger, 2010) through degradation of the mTOR inhibitor, tuberous sclerosis complex 2 (TSC2) in an E6AP-dependent manner (Lu *et al*, 2004b; Zheng *et al*, 2008). This can subsequently activates NF- $\kappa$ B (Basseres & Baldwin, 2006) and inhibits the apoptotic effect of the TNF receptor family (Dolcet *et al*, 2005; Hayden & Ghosh, 2004). Other signalling pathways manipulated by E6s include repression of Notch and enhancement of MAPK signalling pathways (Talora *et al*, 2002a; Chakrabarti *et al*, 2004; Tan *et al*, 2012; Meyers *et al*, 2013).

#### 1.4.3 Biological Synergy of E6 and E7 oncoproteins

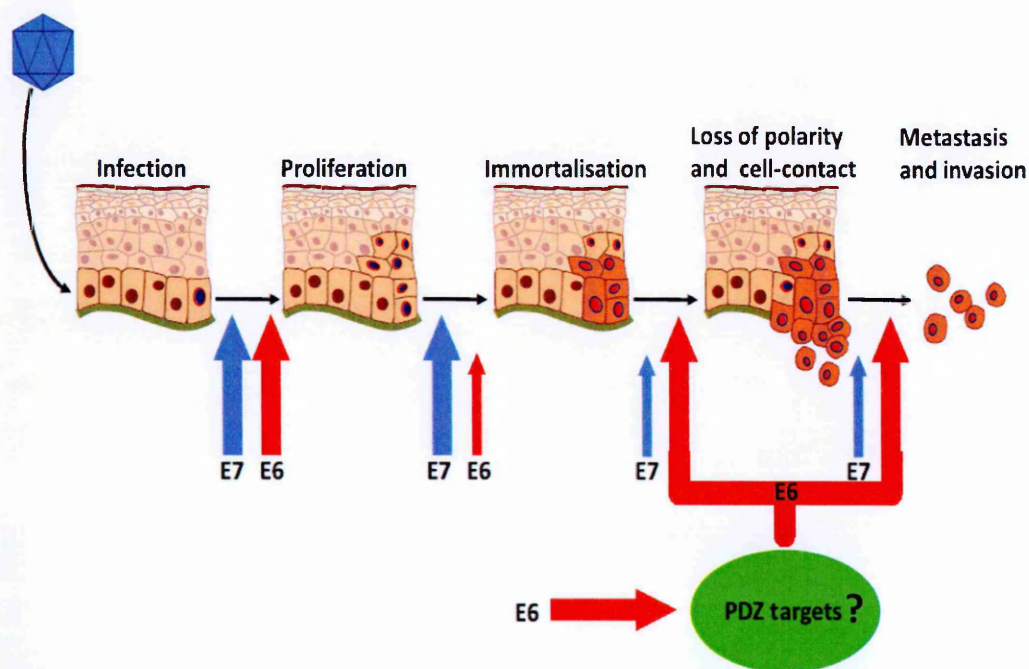
Early studies initially showed that the HR HPVs have the capacity to transform established rodent cells using a variety of different assay system, including NIH 3T3, C127 and 3Y1 (Tsunokawa *et al*, 1986; Yasumoto *et al*, 1986; Morgan *et al*, 1988; Watanabe & Yoshiike, 1988) and mammalian fibroblasts (Watanabe *et al*, 1989). Further studies then demonstrated that HPVs could also cooperate with activated oncogenes, such as EJ-ras (Storey

*et al*, 1988) and c-myc (Crook *et al*, 1989), to bring about full transformation of primary rodent cells. A consequence of the co-expression of HPV oncogenes and activation of ras genes is increased cell density, and the primary rodent cells presented with transformed morphology (Matlashewski *et al*, 1987). Dissection of the viral gene products responsible for these activities indicated a major role for the viral E7 oncoprotein (Storey *et al*, 1988; Phelps *et al*, 1988; Vousden *et al*, 1988), with somewhat weaker activities for the E6 oncoprotein (Hawley-Nelson *et al*, 1989; Sedman *et al*, 1991). Most significantly, in the majority of the cases, these activities were specific for the E7 oncoproteins derived from cancer causing HPV types (Storey *et al*, 1988).

The most relevant cell types to be used for assessing the transforming capabilities are obviously the natural target cells of the virus, human keratinocytes. Early studies demonstrated that HR HPVs could efficiently immortalise human keratinocytes, whether they were derived from either cervical or foreskin demonstrating that HPV had the potential to initiate events leading to transformation in cells derived from multiple locations where the viruses had been associated with the development of malignancy (Dürst *et al*, 1987; Pirisi *et al*, 1987; Kaur & McDougall, 1988; Schlegel *et al*, 1988; Woodworth *et al*, 1988, 1989). It should be emphasized that these immortalized cells were not fully transformed. Extended passages with additional activated oncogenes and the deactivation of tumour suppressors, was required to bring about a fully transformed phenotypes (Band *et al*, 1991; Hurlin *et al*, 1991; Wazer *et al*, 1995; Nakagawa *et al*, 1995). This demonstrates the requirement for other events in addition to the expression

of E6 and E7 for the development of malignancy. These observations reflect the fact that HPV-induced carcinogenesis is a lengthy multi-step event.

Further analyses showed that the ability of HPV to immortalise primary human cells required the combined activity of both E6 and E7, and thus reflected the situation *in vivo*, where both E6 and E7 continue to be expressed in the HPV-induced tumours. The initial findings demonstrated that E7 contributes to increased cell density and transformed morphology, whereas E6 was responsible for the tumorigenicity (Yutsudo *et al*, 1988) and this relative role of both HPV oncoproteins is illustrated in Figure 4. Following this observation, the mechanistic targets of both of these oncoproteins became more apparent. E7 appears to be involved in the early stage of malignant progression, where it induces cell proliferation by targeting pRB and the related pocket proteins, p107 and p130 (Dyson *et al*, 1989; Imai *et al*, 1989; Münger *et al*, 2004). Meanwhile, E6 targets p53 (Scheffner *et al*, 1991; Huibregtse *et al*, 1991; Scheffner *et al*, 1993) and contributes to the progression of the malignancy (Song *et al*, 2000; Riley *et al*, 2003). These results showed that manipulation of these tumour suppressors by HPV oncoproteins is an essential step for HPV-induced tumour progression. Indeed, studies showed that in HPV-negative cervical cancer cell lines isolated from patients, p53 and pRB are mutated and the consequences of these mutations may resemble the effects seen when the wild type tumour suppressors are targeted by the HPV oncoproteins for degradation (Scheffner *et al*, 1991). Additionally, downregulation of E6 and E7 by either expressing viral E2 or using siRNA can reactivate cellular



**Figure 4: Relative contributions of HPV E6 and E7 towards the development of malignancy.** The early stages in the development of cancer are largely driven by the highly proliferative activity of the E7 oncoprotein. The later stages of malignant progression are probably influenced heavily by the E6 oncoprotein and its capacity to target cellular PDZ domain-containing proteins (Pim *et al*, 2012).



tumour suppressive pathways and inhibit HPV-induced immortalisation (Alvarez-Salas *et al*, 1998; Goodwin & DiMaio, 2000; Yoshinouchi *et al*, 2003). In order to understand the biological consequence of HPV E6 and E7 expression *in vivo*, the HPV genome can be introduced into either organotypic raft culture or into animal models.

#### 1.4.3.1 Organotypic raft culture

Organotypic raft culture can be used to study the viral life cycle, HPV-induced immortalisation of keratinocytes, and the phenotypic changes that could occur during differentiation and stratification of the epithelium (Pim & Banks, 2010). To initiate an organotypic HPV infection, the HPV genome is introduced into the primary keratinocytes in monolayer culture. These cells are then grown on a collagen matrix at the air-liquid interface, allowing the cells to differentiate and stratify (Meyers & Laimins, 1994; Chow & Broker, 1997). This differentiation supports the expression of the different viral gene products, ultimately resulting in the production of new viral particles, thereby allowing analysis of the viral life cycle, from initiation of infection to the synthesis of new viral particles. As mentioned earlier, E6 and E7 are involved in manipulating multiple events and affects a huge number of transcripts. Indeed, microarray analysis of primary human keratinocytes containing HPV-18 E6/E7 from an organotypic culture indicates that the viral oncoproteins regulate the expression of a multiple of cellular genes. Upregulated transcripts include the host transcription co-activator, pCAF; transcripts in the MAPK signalling pathways, genes

involved in DNA replication, including cell cycle kinases Cdk2/Cyclin B and Cdk2/Cyclin E; as well as genes involved in the spindle and G2/M checkpoints. Meanwhile, downregulated transcripts include proto-oncogene JunB and genes involved in protein translation (Garner-Hamrick *et al*, 2004). This further highlights the involvement of E6 and E7 in manipulating, directly or indirectly, the expression and functions of a global array of cellular genes and their respective gene products.

One of the benefits of using raft culture is that the morphology of the normal cells as they progress to neoplasia following HPV infection can be observed. Normal epithelial cells show well-defined strata; as the dysplasia progresses following HPV infection, the thickness of the epithelial layers increases with disruption of tissue architecture, irregular size and shape of the individual cells, enlarged and irregular size of nuclei, as well as abnormal mitoses (Blanton *et al*, 1991). The LR HPV types can also alter cell differentiation in raft culture, although immortalisation is extremely rare, hence for studies on LR HPV types E6, normal immortalised keratinocytes (NIKS) are often used. It has also been shown that the integrity of the viral oncogenes is important to establish a stable viral episomal copy number (Hummel *et al*, 1992; Thomas *et al*, 1999; Oh *et al*, 2004b; Delury *et al*, 2013).

#### 1.4.3.2 Animal models - Transgenic mouse models

In addition to raft culture, a number of animal models are used to study the consequences of HPV infection, including the transgenic mouse, canine and cottontail rabbit models. Among these animal models, transgenic mouse

models are widely used to study tumour development induced by E6 and E7. Arbeit *et al.* showed that expression of HPV-16 E6 and E7 induced hyperproliferation of neuroepithelial cells in transgenic mice as early as 1 month old, which further progressed to neuroepithelial tumours (Arbeit *et al.*, 1993). When HPV E6 and E7 were expressed from the keratin 14 (K14) promoter, which drives expression in basal cells (Arbeit *et al.*, 1994), tumours formed at multiple epidermal and squamous sites including skin and cervix (Arbeit *et al.*, 1994, 1996).

Consistent with the observations using primary cells, HPV E6 (Song *et al.*, 1999) and E7 (Herber *et al.*, 1996) are individually competent at inducing epithelial hyperplasia and tumours in animal models. Skin tumours resulting from E7 expression were benign and highly differentiated, whereas E6-induced tumours exhibit more malignant phenotypes (Song *et al.*, 2000; Simonson *et al.*, 2005). Meanwhile, in head and neck squamous cell cancer (HNSCC), E7 plays a more important role than E6 in causing carcinogenesis with increased DNA synthesis (Strati & Lambert, 2007). In the E7-expressing mice, E7 inactivates tumour suppressor pRB (Balsitis *et al.*, 2006), increases proliferation and centrosome copy number, as well as inducing progression of multifocal microinvasive cervical cancers (Riley *et al.*, 2003). However, by using mice expressing a mutant pRB incapable of binding E7, it was found that inactivation of pRB was not sufficient for E7's ability to induce DNA synthesis and overcome differentiation-induced cell cycle arrest, indicating other activities of E7 are also important (Balsitis *et al.*, 2006).

In the case of E6, degradation of p53 is important to induce epidermal hyperproliferation and circumvent p53-induced apoptosis. However, p53-independent activities of E6 are also required (Song *et al*, 1999, 1998). In order to investigate the role of E6AP, E6<sup>I128T</sup>, in which binding to E6AP is strongly reduced (Nguyen *et al*, 2002b), and E6AP-null mice were also used (Shai *et al*, 2007b, 2010). The p53 protein levels are detectable in the mutant E6 mice, and the mice are irresponsive to a radiation-induced block of DNA synthesis (Nguyen *et al*, 2002b). These mice also display reduced epithelial hyperplasia and lower rate of tumour progression (Nguyen *et al*, 2002b) and develop smaller tumours (Shai *et al*, 2007a) in comparison with mice carrying wild type E6. Interestingly, in E6AP-null mice, E6 is able to abrogate the DNA damage response induced by ionizing radiation (Shai *et al*, 2007b, 2010) and can induce epithelial hyperplasia (Shai *et al*, 2007b, 2010). Indeed, studies indicated E6 can also induce hyperplasia through its C-terminal PBM. For example, mice expressing the E6 $\Delta$ PBM ( $\Delta$ 146-151) retain the ability to degrade p53, but exhibit a reduction in DNA synthesis in the suprabasal layer and display reduced tumour sizes (Song *et al*, 1998; Nguyen *et al*, 2003; Simonson *et al*, 2005; Shai *et al*, 2007b), indicating the ability of E6 to abrogate p53-mediated functions, and the involvement of E6's PBM in cancer progression is also depicted in Figure 4.

*In vivo* studies strongly suggest that long-term exposure to estrogen results in HPV-induced invasive cervical carcinoma. Estrogen receptors can be found on the cervical squamous epithelium (Gao *et al*, 1983; Chung *et al*, 2010). Therefore, the cells are able to respond to estrogen treatment, giving rise to hyperplasia and a shorter G1 phase of the cell cycle (Quarmby &

Korach, 1984; Arbeit *et al*, 1996). Transgenic mice expressing E6 and/or E7 showed that estrogen contributes to persistent and continuous development of cervical cancer (Brake & Lambert, 2005; Chung *et al*, 2013, 2008a). HPV infection in combination with the chronic exposure to estrogen and activation of estrogen receptor ER $\alpha$  enhances the effects of cell hyperproliferation (Bradlow *et al*, 1985; Arbeit *et al*, 1996; Chung *et al*, 2013, 2008a; Chung & Lambert, 2009; Son *et al*, 2014; Spurgeon *et al*, 2014). Inhibition of estrogen receptor using antagonists, like raloxifene and fulvestrant, have been proposed to have therapeutic potential for reversing the progression of cervical lesions to carcinoma (Chung & Lambert, 2009; Spurgeon *et al*, 2014).

#### 1.4.4 The E6-PBM and PDZ proteins

One of the unique features of the E6 oncoprotein of the HR HPV types is its ability to bind proteins containing PDZ domains (Postsynaptic density protein 95/Disc large protein/Zonula occludens-1 or in short, PSD95/Dlg/ZO-1). The E6 proteins possess a PDZ binding motif (PBM), x-T/S-x-L/V (Songyang *et al*, 1997) at the C-terminus and this feature is absent in E6s from low risk HPV types. PDZ domains, which are 80 to 100 amino acids in length, are often found in multiple copies within a protein and are the site where protein-protein interactions occur (Fanning & Anderson, 1999). They play significant roles in multiple cellular functions, including regulating cell migration and invasion, cell proliferation, maintaining cell polarity, cell-cell contact, apoptosis, immune cell

recognition and cell signalling (Jacob *et al*, 1987; Woods & Bryant, 1991; Bilder, 2000, 2004; Subbaiah *et al*, 2011) and many of these cellular proteins have potential roles as tumour suppressors (Javier, 2008). E6 targets multiple PDZ proteins through its PBM, and these include human homologue of the *Drosophila* Disc large protein (hDlg) (Kiyono *et al*, 1997; Lee *et al*, 1997), the Scribble (Nakagawa & Huibregtse, 2000) and the membrane-associated guanylated kinase inverted (MAGI) proteins (Glaunsinger *et al*, 2000).

hDlg was the first PDZ protein to be identified as a target for HR HPV E6 (Kiyono *et al*, 1997; Lee *et al*, 1997). hDlg is a member of the membrane-associated guanylate kinase (MAGUK) family of proteins. MAGUK contain PDZ domains, src-homology 3 (SH3) or WW domains and guanylate kinase (GUK) homology domains, all of which can act as modules for protein-protein interactions (González-Mariscal *et al*, 2000; Gardoni *et al*, 2009). These proteins can be found at junctions between epithelial cells where they maintain the integrity of the cell junctions, the localisation of basolateral membrane proteins and apico-basal cell polarity (Woods & Bryant, 1991, 1993; Woods *et al*, 1996). Functionally, they can act as scaffold proteins, binding to transmembrane proteins and bridging signals from the plasma membrane to the downstream effector proteins, and are implicated in diverse activities, including the maintenance of cell polarity and cell signalling (Anderson, 1996). They are also involved in nuclear-cytoplasmic shuttling mediated by the actin cytoskeleton (Islas *et al*, 2002; Jaramillo *et al*, 2004; Traweger *et al*, 2003), and control the kinetics of actin polymerization

(Baumgartner *et al*, 2008) and depolymerisation (Shen & Turner, 2005). Loss or mislocalisation of the PDZ proteins that exist as complexes at the cell junction often results in loss of cell polarity and can contribute to carcinogenesis (Gateff & Schneiderman, 1974; Mechler *et al*, 1985; Bilder & Perrimon, 2000). The ability of E6 to associate with hDlg is conserved among the HR HPV types (Gardioli *et al*, 1999; Pim *et al*, 2000). hDlg is degraded by E6 in a proteasome dependent manner (Woods *et al*, 1996; Kiyono *et al*, 1997; Gardiol *et al*, 1999; Pim *et al*, 2000), thus contributing to a loss of cell polarity, morphological transformation and proliferation. However, the ability of E6 to target hDlg can be influenced both by its location, and its phosphorylation status (Matsumine *et al*, 1996; Gaudet *et al*, 2000; Narayan *et al*, 2009a).

MAGI-1 is important in the establishment of tight junctions, and can affect cell proliferation, apoptosis and cell signalling. MAGI-1 was originally found colocalised with ZO-1 which is an essential component of the epithelial tight junctions (Dobrosotskaya *et al*, 1997; Van Itallie & Anderson, 1997), and has been reported to also regulate AKT activity through complex formation with PTEN, an activity which is also showed by MAGI-2 and MAGI-3 (Wu *et al*, 2000b, 2000a; Kotelevets *et al*, 2005). HPV E6 recognises MAGI-1 via PDZ domain 1 (Glaunsinger *et al*, 2000) and subsequently targets it for degradation (Kranjec & Banks, 2011; Kranjec *et al*, 2014). Recent studies showed that, re-establishment of MAGI-1 expression in HPV positive cell lines allows the tight junction proteins ZO-1 and PAR-3 to be recruited to the cell-cell contact area (Kranjec *et al*, 2014).

Like MAGI-I protein, MAGI-2 and -3 are also targeted by HR HPV E6 for degradation (Thomas *et al*, 2002). This indicates that MAGI proteins are important for maintaining the integrity of cellular tight junctions, and that these controls are perturbed by E6 binding.

Scribble is a non-MAGUK protein, but is also a potentially important target of HPV E6 (Nakagawa & Huibregtse, 2000). Scribble belongs to the LAP (leucine-rich PDZ domain) family of proteins, containing 4 PDZ domains and a leucine rich region which is crucial for its basolateral localisation (Bryant & Huwe, 2000; Santoni *et al*, 2002; Legouis *et al*, 2003). The Scribble polarity module, which is localised at the basolateral junction of the cell membranes, consists of three proteins, Scribble, hDlg and Lgl (lethal giant larvae). In *Drosophila*, this module is important for the proper morphogenesis of the embryonic epithelia (Bilder & Perrimon, 2000), and loss of Scribble results in changes of cell shape, loss of monolayer organisation and loss of cell-cell junctions (Bilder, 2000; Bilder & Perrimon, 2000; Zhan *et al*, 2008). E6 targets Scribble for degradation through a ubiquitin-mediated mechanism (Nakagawa & Huibregtse, 2000; Thomas *et al*, 2005). Both HPV-16 and -18 E6 can interact with Scribble, but HPV-16 E6 is the more effective (Thomas *et al*, 2005). This is due to minor differences in the amino acid sequence of the PBM between different E6 oncoproteins as shown in Figure 5 (a). Indeed, structure and mutational analyses have precisely defined the contribution of each of the amino acids in the PBM in the interactions with different PDZ targets (Songyang *et al*, 1997; Zhang *et al*, 2006b, 2007; Tonikian *et al*, 2008). In the case of hDlg



and Scribble, swapping the last amino acid L/V of HPV-16 and -18 E6, respectively reverses the preference between these substrate proteins (Thomas *et al*, 2005).

Of mammalian PVs, the other HR type that has been defined is Rhesus papillomavirus, RhPV. It was discovered that like humans, the Rhesus Macaque monkey is a natural host for a papillomavirus in which the infection can be transmitted sexually (Ostrow *et al*, 1990; Wood *et al*, 2004). Rhesus papillomavirus type 1 (RhPV-1) shares the closest sequence similarities with HPV-16 (Ostrow *et al*, 1991) and following RhPV-1 infection, Rhesus Macaques develop cervical neoplasia resembling that caused by HR HPV (Wood *et al*, 2007). Intriguingly, the RhPV-1 E7 protein contains a PBM rather than the E6. The PBM of RhPV-1 E7, like that of HR Scribble, HPV E6, confers interaction with a number of PDZ-containing proteins, including MAGI-2 and MAGI-3 (Tomaić *et al*, 2009a). However, the amino acid sequence of the RhPV-1 E7 PBM is very different from the HR HPV E6 PBM, and in this case, Par-3 seems to be the major target of RhPV-1 E7. Par-3 belongs to the same pathway of cell polarity control as hDlg and Scribble, being essential in the function of tight junctions rather than adherens junctions (Macara, 2004; Humbert *et al*, 2008; Chen & Zhang, 2013). RhPV-1 E7 targets the cell polarity regulator, Par3 for degradation in a proteasome dependent manner, similar to HPV E6 (Tomaić *et al*, 2009a). These results indicate that like HPV E6, RhPV-1 E7 can also potentially perturb the control of cell polarity through its PBM.

Another group of PDZ domain-containing proteins targeted by E6 are tyrosine phosphatases, and these include non-receptor tyrosine phosphatases like PTPN3 and PTPN13. In the case of PTPN3, association with HR HPV E6 oncoproteins results in PTPN3 degradation in a proteasome-mediated pathway which is linked to the ability of E6 to induce cell growth even in the absence of growth factors (Jing *et al*, 2007; Töpffer *et al*, 2007). On the other hand, degradation of PTPN13 by E6 oncoproteins results in the upregulation of survival signalling through MAP kinase (Hoover *et al*, 2009), and this event, in cooperation with *ras* expression leads to anchorage-independent growth (Spanos *et al*, 2008b). Activation of cellular kinases may have important implications for the HPV oncoproteins and their target proteins, potentially affecting their preferences in protein-protein recognition and localisation.

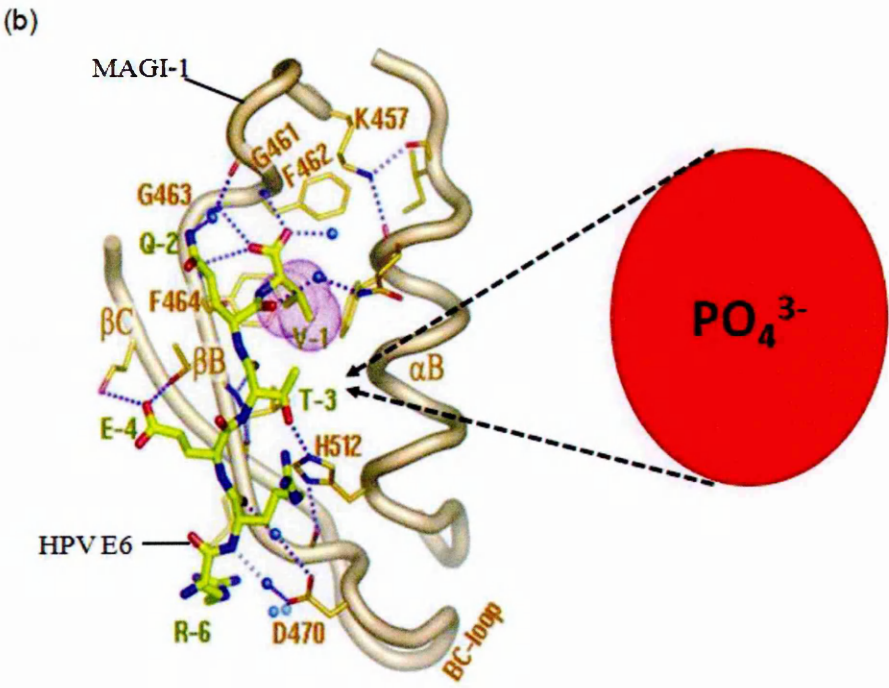
E6's PBM plays a crucial role in viral life cycle and in HPV-induced carcinogenesis. In the viral life cycle, the integrity of E6 and its PBM is crucial for the maintenance of the viral copy number in undifferentiated cells (Lee & Laimins, 2004; Delury *et al*, 2013). Kiyono and colleagues showed that an intact HPV-16 E6 PBM is required to induce morphological transformation in rat 3Y1 cells (Kiyono *et al.*, 1997). Meanwhile, E6 mutants with deletions in the PBM are unable to induce hyperplasia in transgenic mouse models (Nguyen *et al*, 2003) and are also unable to cooperate with E7 to immortalise human tonsillar epithelial cells (Spanos *et al*, 2008b, 2008a). In human foreskin keratinocytes (HFKs), the E6's PBM contributes towards induction of epithelial hyperplasia in raft cultures, and

also aids in the retention of viral episomes over time (Lee & Laimins, 2004; Delury *et al.*, 2013; Choi *et al.*, 2014). PDZ proteins possess dual functions, mostly acting as tumour suppressors, however, they can also have oncogenic properties, depending on the context. For instance, HPV-18 E6 was found to form as a complex with hDlg and the RhoG-specific guanine nucleotide exchange factor (GEF), SGEF, through E6's PBM and this complex increases RhoG activity (Subbiah *et al.*, 2012). Intriguingly, the pool of hDlg that is not degraded by E6, together with SGEF, contributes to invasiveness in HPV-transformed cells. This probably occurs through upregulation of SGEF and RhoG activity (Subbaiah *et al.*, 2012). These findings collectively highlight the importance of the PBM of HR HPV E6 in interacting with PDZ proteins, degrading these proteins and regulating cellular signalling pathways that favour virus survival or cell transformation.

As can be seen in Figure 5 (a), the amino acid sequence of the E6 PBMs of the different HR types of HPV types are similar and this domain can potentially be subject to post-translational modification by phosphorylation. It has been shown that the E6 of HR HPV can be phosphorylated by PKA, but not the E6s from LR types, with the phospho-acceptor on HPV-18 E6 mapping to T156. Kinetic assays on peptides of the different HR HPV E6s indicated that, despite the close similarity of the of the C-terminal PBM of these E6s, their susceptibility to phosphorylation by PKA varies (Kühne *et al.*, 2000). Studies performed by Kuhne *et al.* (2000) showed several important points. HPV-18 E6 can be strongly phosphorylated by PKA compared with PKB, p70S6K and p90rsk. In addition, the Michaelis constant,  $K_m$ , of the HPV-18 and -16 E6s phosphorylated by PKA are

similar, 6.0 and 6.6 respectively, while the  $K_m$  value for HPV-33 E6 was 3.6 (Kühne *et al*, 2000). These indicate that these proteins can be phosphorylated efficiently and specifically by PKA. However, the phosphoregulation of the full length E6 proteins remained to be verified and the susceptibility of the HR HPV E6s to phosphorylation by other kinases remained largely unknown. As the phospho-acceptor residue is embedded within the PDZ recognition region, the effect of phosphorylation in interacting with PDZ proteins was also investigated. Intriguingly, phosphorylation of HPV-18 E6 negatively regulates its interaction with hDlg, hence rescuing hDlg from degradation by E6 (Kühne *et al*, 2000). Structural analysis revealed that the interacting groove between E6 and PDZ proteins is too small to accommodate a phosphate molecule as shown in Figure 5 (b) (Zhang *et al*, 2007). Even though phosphorylation negatively regulates the interaction of E6 with PDZ-containing proteins, it was possible that the post-translational modification of E6 might confer interaction with other cellular proteins and this aspect remained largely to be investigated. However, proteomic analysis performed in our laboratory and by Howie *et al* showed that 14-3-3 proteins (Howie *et al*, 2011), phosphoserine/threonine binding proteins are possible novel interacting partners of phospho-E6 (pE6).

(a)	PKA consensus site:	RRX <b>T/S</b>
	PDZ binding motif:	X <b>T/S</b> XV/L
	HPV E6 types	PBM Sequence
	18	.....RLQRRR <b>ETQV</b> *
	16	.....SSRTRR <b>ETQL</b> *
	33	.....WRSRRR <b>ETAL</b> *
	51	.....RLQRRN <b>ETQV</b> *
	58	....WRPRRR <b>QTQV</b> *
	31	....WRRPRT <b>ETQV</b> *



**Figure 5. The E6 PDZ binding motif (PBM).** (a) Close similarity of amino acid sequence of PBM of E6 across the different cancer-causing HR HPV types E6. This is the region where the potential PKA phosphorylation site embedded within PBM of HPV-18 E6 has been identified. (b) E6 (E155 residue) and PDZ proteins (GFGL motif spanning from amino acid 461 to 464 in MAGI-1 PDZ domain 1) interaction is held by hydrogen bonding (dotted blue line). The hydroxyl group of T3 is only 3Å (0.3 nm) away from αB of the MAGI-1. Adding of a phosphate group (red sphere  $\text{PO}_4^{3-}$ ) by PKA phosphorylation at T156 of E6 creates a steric hindrance that prevents E6 from interacting with MAGI-1 (Adapted from Zhang *et al.*, 2007).

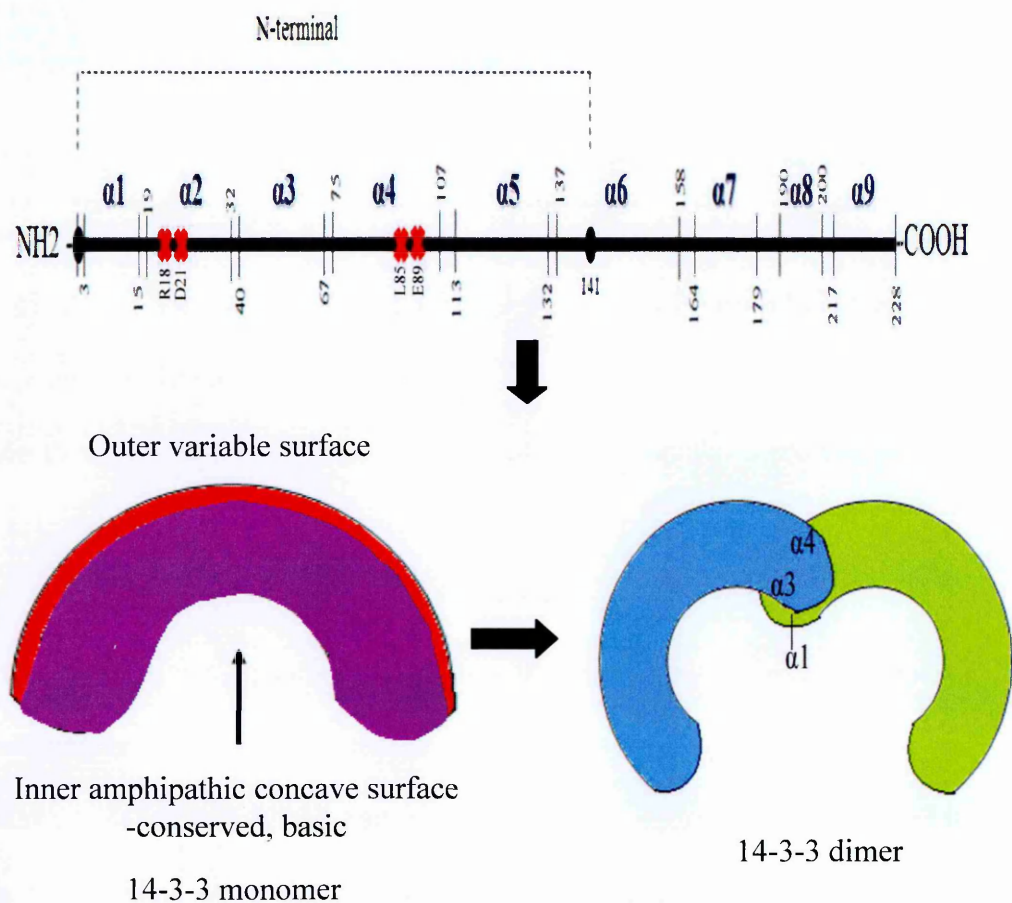
### 1.5 14-3-3 genes, protein structure and their general cellular functions

14-3-3 proteins were first discovered in 1967 from a fractionation analysis of soluble proteins isolated from brain (Moore & Perez, 1967). The nomenclature of these proteins was given according to the combination of their fraction number on DEAE-cellulose chromatography and their migration pattern on electrophoresis (Moore & McGregor, 1965). 14-3-3 proteins are highly conserved in most eukaryotes including mammals, amphibians, insects and plants (Aitken *et al*, 1992), and seven isoforms of 14-3-3 have been identified in mammals, namely  $\alpha/\beta$ ,  $\gamma$ ,  $\epsilon$ ,  $\zeta/\delta$ ,  $\eta$ ,  $\tau/\theta$  and  $\sigma$  (Mackintosh, 2004), which are encoded by distinct genes located on different chromosomes (Aitken, 2002).

14-3-3 proteins are highly acidic, with isoelectric points of 4 to 5 and molecular weights of 25 to 33 kDa (Jones *et al*, 1995a) and they are ubiquitously expressed in cells. They are also extremely abundant: it was estimated that 14-3-3 protein made up 1% of the brain soluble protein (Boston *et al*, 1982). Although it was initially found in brain tissue, 14-3-3 was later found to be present in almost all tissues analysed (Celis *et al*, 1990). In terms of its intracellular distribution, 14-3-3 is found predominantly in the cytoplasm, but it is also found in plasma membranes, nucleus and Golgi apparatus (Celis *et al*, 1990; Leffers *et al*, 1993; Tang *et al*, 1998; Freed *et al*, 1994; Garcia-Guzman *et al*, 1999; Fanger *et al*, 1998).

The crystal structures of the  $\tau$  and  $\zeta$  isoforms showed that 14-3-3 are dimeric proteins. Each of the monomers contains variable N-terminal and conserved C-terminal domains, with the 9  $\alpha$ -helices arranged in anti-parallel manner

(Liu *et al*, 1995; Xiao *et al*, 1995). Figure 6 shows the arrangement of the 14-3-3 dimer, and as can be seen, the molecule is orientated in such a way that it has a cup-like structure. Its outer surface is variable whilst the inner amphipathic concave surface is highly conserved. The dimer is formed through packing of helices  $\alpha 1$  (amino acid residue 3 - 15) of one monomer with helices  $\alpha 3$  (around amino acid 40 - 67) and  $\alpha 4$  (around amino acids 75 - 107) (Liu *et al*, 1995; Xiao *et al*, 1995). The dimer interface forms a negatively-charged channel (Aitken, 2002; Xiao *et al*, 1995), and the dimer is held together by salt bridges (Liu *et al*, 1995; Yang *et al*, 2006). This fundamental structure of 14-3-3 and dimerisation are also illustrated in Figure 6. Due to the specificity with which the monomers can interact, each isoform is restricted in how it can form homo- or heterodimers with the other 14-3-3 isoforms (Jones *et al*, 1995b). When 14-3-3 binds to the ligand, the ligand runs in the opposite direction within the amphipathic binding groove of the 14-3-3 dimers (Jones *et al*, 1995a; Fu *et al*, 2000). This dimer formation and ligand protein binding is illustrated in Figure 7. Only specific combinations of 14-3-3 isoforms form dimers, and this may confer some kind of specificity upon 14-3-3 function. This will be discussed in a later section.



**Figure 6: Basic molecular structure of 14-3-3.** The 14-3-3 monomer contains 9  $\alpha$ -helices and the protein is folded in such a way that the variable surface is facing out (red) and the amphipathic concave surface which is conserved among the isoforms is facing inward (purple). The dimer interface results from the packing of the  $\alpha$ -1 monomer (blue) with  $\alpha$ 3 and  $\alpha$ 4 helices of the other (green). The 14-3-3 $\zeta$  homodimer is held together at R18 and E21 (embedded within  $\alpha$ 2 helix), as well as L85 and M88 (embedded within  $\alpha$ 4 helix) through salt bridges (red crosses).

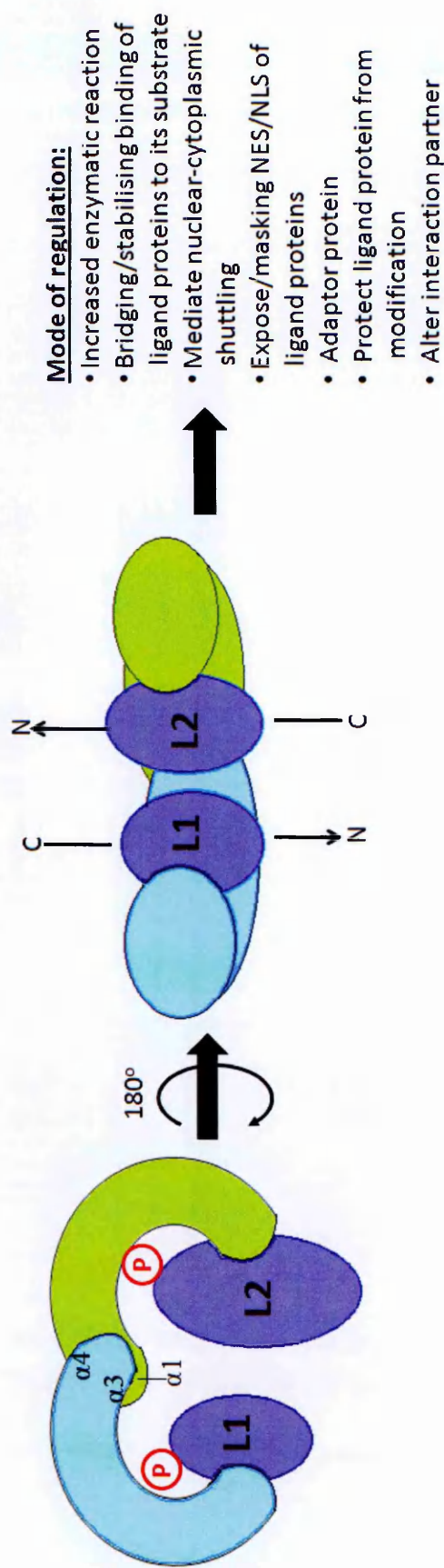


## 1.6 14-3-3 ligand protein recognition and consequences of the association

14-3-3 binds to over 100 cellular proteins and many of these interactions occur in a phosphorylation-dependent manner. Although 14-3-3 can also bind to non-phosphorylated proteins, the number of these interactions are fewer than those with phosphorylated proteins. From co-crystallization analysis of 14-3-3 $\zeta$  bound to different peptide ligands, it was shown that binding of both phosphorylated and non-phosphorylated peptides to 14-3-3 occurs within the conserved concaved amphipathic groove (Yaffe *et al*, 1997; Rittinger *et al*, 1999; Petosa *et al*, 1998). 14-3-3 can alter the functions or nuclear-cytoplasmic localisation of its bound ligand proteins; it can either protect it from, or promote its susceptibility to further post-translational modifications and affect its ability to interact with other cellular proteins (Hermeking, 2003). Binding of 14-3-3 to the ligand proteins and the mode of regulation by 14-3-3 are also illustrated in Figure 7.

### 1.6.1 Phosphoprotein recognition

All 14-3-3 isoforms are known to be phospho-serine/threonine binding proteins (Muslin *et al*, 1996). They recognise the consensus motifs RSXpSXP, RXY/FXpSXP and p(S/T)X<sub>1-2</sub>-COOH and these binding motifs are present in many of the 14-3-3 binding proteins (Yaffe *et al*, 1997; Coblitz *et al*, 2006). The binding site for the phosphoserine/threonine proteins is the basic pocket of 14-3-3 consisting of Lys-49 (K49), Arg-56 (R56), Arg-127 (R127), and Tyr-128 (T128). Cellular proteins which are recognised as phosphorylated proteins by 14-3-3 include protein kinases



**Figure 7: Binding of 14-3-3 dimer to phosphorylated ligand protein and mode of regulation upon association to 14-3-3.** 14-3-3 dimerised through interaction of  $\alpha 1$  (green) of one of the monomer with  $\alpha 3$  and  $\alpha 4$  (blue) of the other monomer, to form either homodimer (green and light blue are the same monomer) or heterodimer (green and light blue are different monomer). 14-3-3 dimer binds to phosphorylated ligand protein (L1 and L2, where they can be similar or different proteins in purple) in anti-parallel manner. For instance, 14-3-3 $\beta$  or  $\tau$  (light blue) associates with Bcr (L1) and 14-3-3 $\zeta$  or  $\beta$  (green) binds to Raf (L2). The association with 14-3-3 enables 14-3-3 to regulate functions of the bound ligand proteins.

(murine leukemia viral oncogene homologue - RAF1, MEK kinase, PI3 kinase, and Grb10), growth factor receptor proteins (insulin-like growth factor 1 and glucocorticoid receptors), enzymes (serotonin N-acetyltransferase, tyrosine and tryptophane hydroxylase), structural and cytoskeletal proteins (vimentins, and keratins), scaffolding molecules (calmodulin), proteins involved in cell cycle control (Cdc25, p53, p27, and Wee1), proteins involved in transcriptional control (histone acetyltransferase, and TATA box binding proteins), and proteins involved in apoptosis (Bad) (Yaffe *et al*, 1997; Aitken, 2002; Fu *et al*, 2000; Mhawech, 2005).

### 1.6.2 Unmodified protein recognition

As mentioned above, 14-3-3 can also interact with unmodified proteins (Hermeking, 2003; Mackintosh, 2004). Examples of these include mitochondrial targeting signal sequences (Alam *et al*, 1994), the platelet glycoprotein, GPIIb (Du *et al*, 1996), the ExoS ADP-ribosyltransferase (Fu *et al*, 1993; Masters *et al*, 1999), inositol polyphosphate 5-phosphatase (5-phosphatase) (Campbell *et al*, 1997), the pro-apoptotic protein Bax (Nomura *et al*, 1999), as well as Raf-1 at its cysteine rich domain (CRD) (Michaud *et al*, 1995; Clark *et al*, 1997). Unlike the phosphoserine/threonine binding motif, 14-3-3 recognition of unmodified ligands seems to depend upon the presence of multiple negatively-charged residues, such as Glutamic acid (E) or Aspartic acid (D). For example, 14-3-3 recognises the RSxSxP-like motif RSESEE, which is located close to the catalytic domain of 5-phosphatase (Campbell *et al*, 1997). Similarly, a small 18-amino acid peptide, R18,

which contains multiple negatively charged E residues can also bind to 14-3-3 and act as an antagonist (Wang *et al*, 1999). Interestingly, the interactions of 14-3-3 with non-phosphorylated ligands can occur with high affinity, for example the 13-mer peptide of 5-phosphatase containing the RSESEE motif has a  $K_d$  (dissociation constant) value of 92 nM (Campbell *et al*, 1997). In contrast, p53 peptides mono-phosphorylated at S366, S378 or T387 bind to 14-3-3 with a  $K_d$  of 14 - 20  $\mu$ M (Rajagopalan *et al*, 2008), whilst 14-3-3 $\zeta$  binds to the Raf-1 peptide pS259 with a  $K_d$  of 7.5  $\mu$ M (Molzan *et al*, 2010).

### 1.6.3 14-3-3 regulation of its target proteins

14-3-3 interactions with its target proteins can have a wide variety of effects, depending upon the particular context. In the case of enzymes, this may involve stimulation of enzymatic reaction. For instance, association of the 14-3-3 dimer with Raf-1, increases its intrinsic serine/threonine kinase activity (Morrison *et al*, 1993; Tzivion *et al*, 1998). Likewise, association of 14-3-3 with a serotonin N-acetyltransferase also enhances its enzymatic activity (Obsil *et al*, 2001).

14-3-3 can also mediate nuclear-cytoplasmic shuttling of its target proteins (Muslin & Xing, 2000). In most cases, 14-3-3 mediates localisation of its target protein to the cytoplasmic compartment. For instance, the cell cycle regulator, Cdc25 also associates with 14-3-3, and as a consequence, Cdc25 is relocalised into the cytoplasm as an inactive form (Dunphy & Kumagai, 1991; Lopez-Girona *et al*, 1999; Yang *et al*, 1999; Uchida *et al*, 2004c),

keeping it away from cyclin-dependent protein kinase (Cdk) complexes (Chou *et al*, 2010). It was initially thought that the cytoplasmic relocalisation of 14-3-3-ligand complexes was due to the presence of a putative nuclear export sequence (NES) in the C-terminal region of 14-3-3 (Lopez-Girona *et al*, 1999; Rittinger *et al*, 1999), but several studies now appear to disagree with this (Yang *et al*, 1999; Zeng & Piwnica-Worms, 1999; Kumagai & Dunphy, 1999; Brunet *et al*, 2002). For example, it was shown that a 14-3-3 mutant which is not able to bind to its ligand accumulates in the nucleus (Brunet *et al*, 2002). This is thought to be due to the fact that the 14-3-3-ligand interaction results in a conformational change of the target protein, which in turn facilitates an alteration in cellular localisation, depending on the exposure of an NES or NLS on the target protein. Hence, 14-3-3 may serve as a chaperone protein (Brunet *et al*, 2002; Muslin & Xing, 2000). For instance, FKHRL1 when phosphorylated by AKT binds to 14-3-3, and this confers a structural change which exposes 2 NESs within FKHRL1. At the same time, this association also masks the nuclear localisation sequence (NLS) of FKHRL1 and prevents its relocalisation into the nucleus, thereby, maintaining FKHRL1 in an inactive form in the cytoplasm (Brunet *et al*, 2002). Other examples of proteins which are sequestered by 14-3-3 into cytoplasm in their inactive form include histone deacetylase 4 (HDAC4) and HDAC5 (Grozinger & Schreiber, 2000; Ellis *et al*, 2003), high-mobility-group nucleosomal-binding protein 1 (HMGN1) (Prymakowska-Bosak *et al*, 2002), transcription co-activator in Hippo pathways, TAZ (Kanai *et al*, 2000), transcription factor DAF16 (Cahill *et al*, 2001) and p27 (Fujita *et al*, 2002).

14-3-3 can also serve as an adaptor protein where it bridges 2 proteins that do not interact directly. A classic example of this is again provided by Raf-1 where interaction with 14-3-3 bridges the interaction with Bcr (Brasemann & McCormick, 1995), protein kinase C- $\zeta$  (PKC- $\zeta$ ) (Van Der Hoeven *et al*, 2000) and a zinc finger protein, A20 (Vincenz & Dixit, 1996) through 14-3-3 dimers. 14-3-3 interactions can also alter the susceptibility of the bound protein to further post-translational modifications, for example, binding of 14-3-3 to Bad (Chiang *et al*, 2001) and tristetraprolin (TTP) (Sun *et al*, 2007; Kraemer & Clement, 2014), a zinc finger RNA binding protein, protecting these proteins from dephosphorylation.

### 1.7 Regulation of 14-3-3 by phosphorylation

The role of 14-3-3 in regulating the function of its binding partners has been discussed. However, 14-3-3 proteins themselves are also subject to regulation, mainly by phosphorylation, which in turn can affect 14-3-3 dimerisation. From the structural analyses, it was predicted that under physiological conditions where the majority of 14-3-3 exists as a dimer, it might be difficult for a kinase to gain access to the phosphorylation sites. This is because the 14-3-3 dimers are very stable under normal conditions (Aitken, 2002; Shen *et al*, 2003). However, numerous studies have shown that 14-3-3 can be phosphorylated at residues S58, S59, S64, S185 and S233 by several different kinases. For example, S58 can be phosphorylated by PKA, AKT or mitogen-activated protein kinase (MAPK)-activated protein kinase 2 (MAPKAPK2). It was initially shown that under *in vitro*

conditions, 45% of 14-3-3 $\zeta$  is phosphorylated by AKT at S58, a site embedded within the dimerisation interface and this phosphorylation does not affect 14-3-3 dimerisation (Powell *et al*, 2002). Later studies contradicted this with the observation that PKA phosphorylation of 14-3-3 at S58 affects its dimerisation and affects its functional interaction with p53 (Gu *et al*, 2006). Like PKA, phosphorylation of 14-3-3 at S58 by MAPK-activated protein kinase 2, MAPKAPK2, also inhibits the dimerisation of 14-3-3 and its subsequent interaction with Raf-1 (Powell *et al*, 2003).

14-3-3 proteins are also subject to phosphorylation by Casein kinase I (CKI) at T233 in 14-3-3 $\zeta$  and S233 in 14-3-3 $\tau$ . This residue is also implicated in 14-3-3 target recognition. In the case of Raf-1 recognition, phosphorylation of 14-3-3 at this site inhibits the interaction, and thereby has important implications for the Raf-1 signal transduction pathway (Dubois *et al*, 1997b, 1997a). Other kinases shown to regulate 14-3-3 include protein kinase C (PKC) and sphingosine-dependent protein kinase (SDK1) with different PKC isoforms phosphorylating different 14-3-3 isoforms. For instance, PKC- $\zeta$  can phosphorylate 14-3-3 $\beta$  and  $\tau$  (Van Der Hoeven *et al*, 2000), whilst 14-3-3 $\gamma$  can be phosphorylated by PKC $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\theta$  and  $\delta$  isoforms (Autieri & Carbone, 1999). SDK1 can phosphorylate 14-3-3 $\eta$ ,  $\beta$  and  $\zeta$ , but not the  $\sigma$  and  $\tau$  isoforms (Megidish *et al*, 1998). Taken together, these studies show that phospho-regulation of 14-3-3 is highly complex, and varies considerably between the different isoforms. However, in all cases, it has important implications for 14-3-3 regulation and function.

### 1.8 The 14-3-3 Heterodimer

14-3-3 can exist as either homo- or heterodimers. Although not all isoforms can heterodimerise with each other and there is a clear preference for specific 14-3-3 isoforms in each heterodimer (Jones *et al*, 1995a). The preferential combinations are listed in Table 3. It is important to note that, once formed, the heterodimers are stable and binding partners are not readily exchanged (Jones *et al*, 1995a).

Studies using overexpression of different 14-3-3 isoforms revealed distinct preferences for particular monomer combinations that are largely independent of the cellular conditions. It was found that 14-3-3 $\gamma$  can exist as a homodimer but also formed heterodimers, mainly with 14-3-3 $\epsilon$  (Chaudhri *et al*, 2003). Other studies found that, besides dimerising with  $\epsilon$ , 14-3-3 $\gamma$  also weakly dimerised with the  $\zeta$  and  $\beta$  isoforms (Jones *et al*, 1995a). In addition,  $\tau$  can dimerise with the  $\zeta$  isoform, whilst  $\sigma$  isoform preferentially exists as a homodimer (Wilker *et al*, 2005; Chaudhri *et al*, 2003). As noted above, the ability of 14-3-3 to dimerise is a critical aspect of 14-3-3 function, and this is revealed most strongly when it performs a bridging role, as for example with Raf-1 and Bcr. This bridging involves 3 14-3-3 isoforms,  $\beta$ ,  $\tau$  and  $\zeta$ . 14-3-3  $\beta$  and  $\tau$  associate with Bcr while the  $\zeta$  and  $\beta$  interact with Raf (Brasemann & McCormick, 1995) as illustrated in Figure 7, and this brings Raf and Bcr into close proximity.



**Table 3: Summary of the 14-3-3 isoform interactions with other 14-3-3 isoforms to form homo- or heterodimers.** Symbols:  $\checkmark$  indicates that the monomers can dimerise, x indicates that the monomers does not bind to each other, whilst ? means that no information is available.

Isoforms	$\zeta/\delta$	$\sigma$	$\gamma$	$\varepsilon$	$\eta$	$\tau/\theta$	$\beta/\alpha$
$\zeta/\delta$	$\checkmark$	x	$\checkmark$	$\checkmark$	?	$\checkmark$	$\checkmark$
$\sigma$	x	$\checkmark$	$\checkmark$	$\checkmark$	?	?	?
$\gamma$	$\checkmark$	?	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$	$\checkmark$
$\varepsilon$	$\checkmark$	?	$\checkmark$	$\checkmark/x$	$\checkmark$	$\checkmark$	$\checkmark$
$\eta$	?	?	$\checkmark$	$\checkmark$	$\checkmark$	?	?
$\tau/\theta$	$\checkmark$	?	$\checkmark$	$\checkmark$	?	$\checkmark$	?
$\beta/\alpha$	$\checkmark$	?	$\checkmark$	$\checkmark$	?	$\checkmark$	$\checkmark$

### 1.9 14-3-3 regulates the Ras-Raf-MAPK pathway

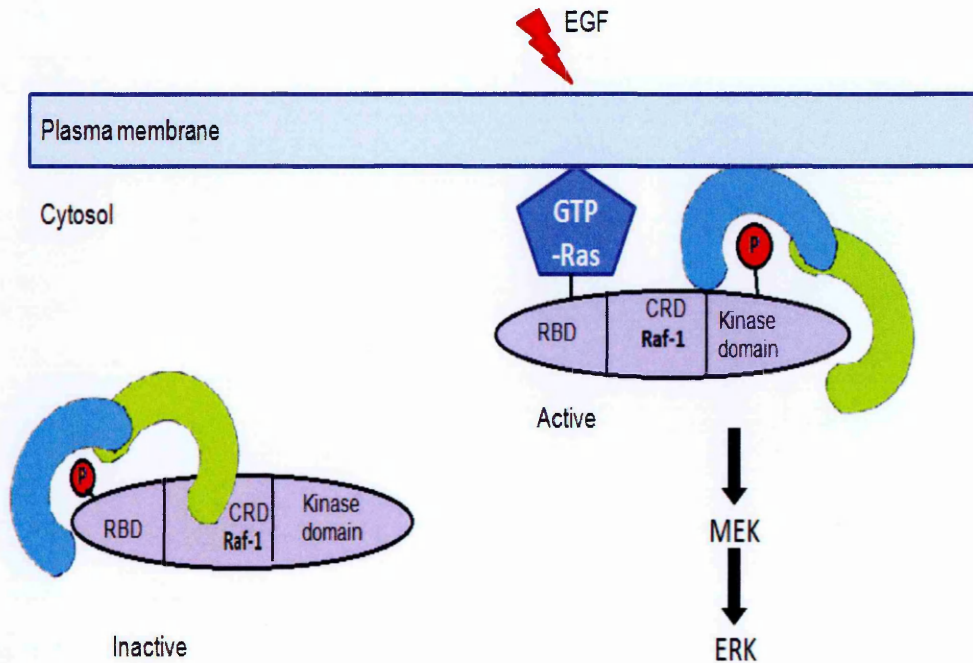
The activation of Raf-1 is regulated by 14-3-3 (Fu *et al*, 1994; Freed *et al*, 1994). Structurally, Raf-1 is divided into an N-terminal Ras binding domain (RBD), CRD and a C-terminal kinase domain. 14-3-3 can bind to the phospho-acceptor residues of Raf-1 at S233, S259 and S621 in the C-terminal kinase domain (Morrison *et al*, 1993; Muslin *et al*, 1996; Rommel *et al*, 1997; Molzan & Ottmann, 2012). The mutation S259A and S621A, which cannot be phosphorylated, abolish the interaction of Raf-1 with 14-3-3, indicating that this association occurs in a phosphorylation-dependent manner (Michaud *et al*, 1995). When both S259 and S621 of Raf-1 are phosphorylated, 14-3-3 will bind to the N-terminus of Raf-1 and this introduces a conformational change in Raf-1 (Dumaz & Marais, 2003). Association of 14-3-3 with Raf-1 can have 2 possible consequences: binding of 14-3-3 to Raf-1 at S233 and S259 inhibits Raf-1 activity by retaining Raf-1 in the cytoplasm; in contrast, binding of 14-3-3 to S621 increases Raf-1

kinase activity. When the cells are unstimulated, Raf-1 is phosphorylated at S233 and S259, creating a 14-3-3 binding motif (Rommel *et al*, 1997; Dubois *et al*, 1997b; Dumaz & Marais, 2003). 14-3-3 keeps Raf-1 in the cytosol in an inactive form, away from Ras and preventing recruitment of Raf-1 to the plasma membrane (Rommel *et al*, 1997; Dumaz & Marais, 2003). Therefore, 14-3-3 plays a critical role in suppressing Raf-1 activation. However, upon stimulation, Ras associates with Raf-1, displacing 14-3-3 from interaction with Raf-1 (Li *et al*, 1995; Michaud *et al*, 1995; Rommel *et al*, 1996, 1997; Light *et al*, 2002). Studies suggested that this displacement might indicate that 14-3-3 is not required for Raf-1 kinase activity (Li *et al*, 1995; Michaud *et al*, 1995; McPherson *et al*, 1999; Roy *et al*, 1998; Light *et al*, 2002). However, other studies disagreed with this notion. It was shown that when the cells is stimulated, 14-3-3 can bind to Raf-1 at S621, which is located in the C-terminal kinase domain of Raf-1, and this association appears to be required for activation of Raf-1 (Dent *et al*, 1995; Dhillon *et al*, 2009; Tzivion *et al*, 1998; Fischer *et al*, 2009). 14-3-3 association at S621 confers a conformational change of Raf-1 and prevents it from dephosphorylation (Dent *et al*, 1995; Thorson *et al*, 1998) and the activated Raf-1, either by Ras or 14-3-3 association at S621, is recruited to the plasma membrane (Marais *et al*, 1995; Stokoe *et al*, 1994). This activation also requires the oncogenic B-Raf, one of the Raf family members, to form heterodimers with Raf-1 mediated by 14-3-3 (Wan *et al*, 2004; Garnett *et al*, 2005; Rushworth *et al*, 2006; Ritt *et al*, 2010). Different 14-3-3 isoforms may play different roles in Raf-1 activity. The expression of 14-3-3 $\gamma$  results in increased Raf-1 activity (Radhakrishnan & Martinez,

2010) and subsequently leads to phosphorylation of kinases involved in the MAPK signalling pathway (Aitken, 2002; Downward, 2003; Galabova-Kovacs *et al*, 2006; Seger & Krebs, 1995). In contrast, 14-3-3 $\sigma$  inhibits these activities (Radhakrishnan & Martinez, 2010). The kinase that phosphorylates the kinase domain of Raf-1 is PKC and this creates a 14-3-3 association with Raf-1 (Kolch *et al*, 1993), and this subsequently leads to activation of survival MAPK signalling cascade, as well as PI3K (Takahara *et al*, 2000; Radhakrishnan & Martinez, 2010). In this way, PKC plays a positive role in activating a member of the MAPK pathway, like MEK (Blumer *et al*, 1994). All these studies indicate that 14-3-3 plays a critical role in mediating the Raf-1 signalling pathway in cell growth, regardless of whether it is active or inactive and this is summarised in Figure 8.

### **1.10 14-3-3 in DNA damage-induced cell cycle checkpoints**

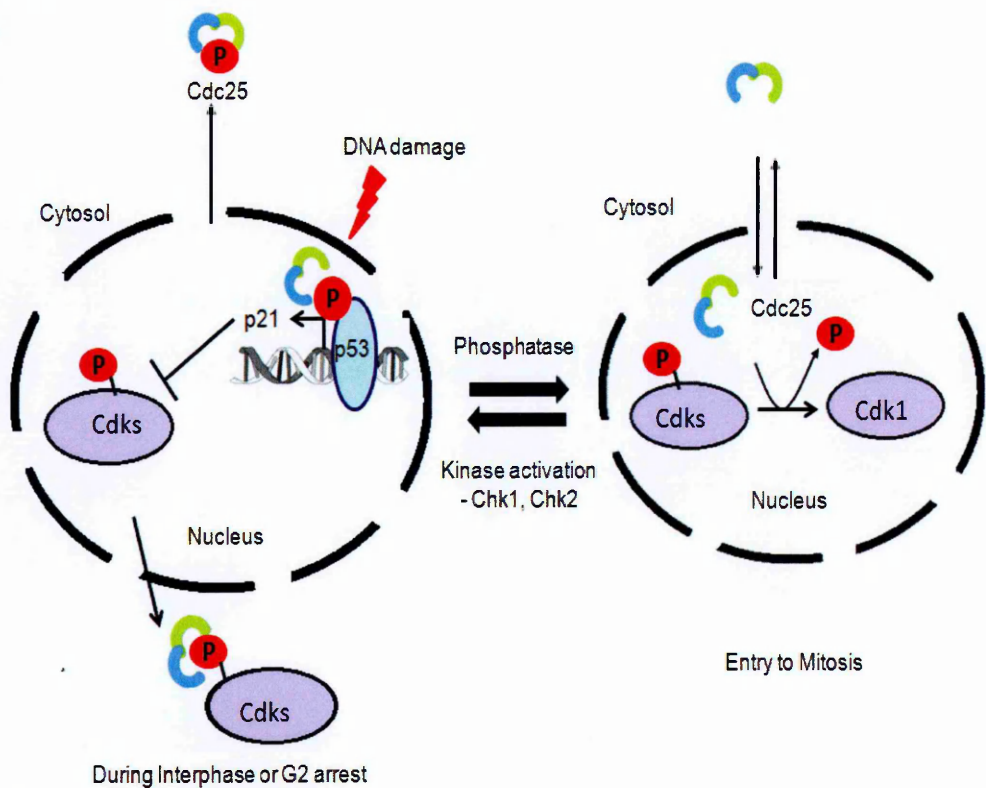
During interphase, 14-3-3 exists predominantly in the cytoplasm, and it enters the nucleus during the mitosis, particularly in the perichromosomal region (Su *et al*, 2001), thus suggesting a role in cell cycle regulation. This regulation occurs through the association of 14-3-3 with components of the cell cycle checkpoint control pathways, including Cdc25 (Cdc25A, B and C), p53, cyclin-dependent kinase (Cdk) inhibitor p27 and tuberous sclerosis complex (TSC) proteins (Hermeking & Benzinger, 2006). In general, when the cell does not have the cell cycle checkpoint activation, these proteins are kept in an inactive form in the cytoplasm by 14-3-3. In the case of Cdc25, it



**Figure 8: The role of 14-3-3 in Ras-Raf-MAPK signalling pathway.** 14-3-3 dimer (in blue and green) associates with Raf-1 in a phosphorylation-dependent manner and regulates Raf-1 function in different cellular compartments. In cytosol, 14-3-3 associates with the N-terminal Ras binding domain (RBD) of phosphorylated Raf-1 and keeps it in an inactive form. Upon receiving cell growth stimuli by epidermal growth factor (EGF), Raf-1 is recruited to the plasma membrane and associates with GTP-Ras. Phosphorylation of the C-terminal kinase domain of Raf-1 by PKC confers association with 14-3-3 and thereby enhances its kinase activity to activate its downstream MEK and ERK pathways. This in turn activates the downstream transcriptional activities which are crucial for cell division.

is phosphorylated at S216, S263 and S247 (Peng *et al*, 1997; Esmenjaud-Mailhat *et al*, 2007; Chan *et al*, 2011) and this confers binding to 14-3-3. As a result, Cdc25 is sequestered by 14-3-3 in the cytoplasm and remains inactive. When the cells enter mitosis, Cdc25 is dephosphorylated by protein phosphatase 2A (PP2A) (Margolis *et al*, 2006; Forester *et al*, 2007), and Cdc25 becomes active; it can then enter the nucleus to dephosphorylate and activate Cdk1/Cyclin B complex, which is the key regulator of the onset of mitosis (Lammer *et al*, 1998; Hoffmann *et al*, 1993; Gautier *et al*, 1991). Activation of Cdk1 then triggers cell cycle to progress to mitosis (Russell, 1998).

When cells encounter cell cycle checkpoint activation as a result of DNA damage, Cdc25 is phosphorylated by TAK or Chk1 at S216 (Peng *et al*, 1998; Liu *et al*, 2000; Chaturvedi *et al*, 1999; Sanchez *et al*, 1997; Peng *et al*, 1997) to generate a 14-3-3 binding motif. As a result, Cdc25 is sequestered in the cytoplasm by 14-3-3, and therefore remains inactive, as it is not able to dephosphorylate the Cdk1/Cyclin B complex, and the cell cycle progression into mitosis is halted (Hermeking & Benzinger, 2006). The involvement of 14-3-3 in regulating cell cycle progression in interphase, DNA damage-initiated G2 arrest, and mitotic entry is shown in Figure 9.



**Figure 9. The role of 14-3-3 in regulating Cdc25 and Cdks during cell cycle.** During interphase or DNA damage induction, Cdc25, Cdks (Cdk2 in G1/S and Cdk1 in G2/M phases) and p53 are subject to phosphorylation by several kinases including Chk1 or Chk2 and this creates a 14-3-3 binding motif. 14-3-3 (light blue and green) association leads to recruitment of Cdc25 and Cdks into the cytosol, thereby maintaining them in an inactive state. Association of p53 with 14-3-3 enhances p53 DNA binding ability and thereby enhances p21 transcriptional activation. p21, can in turn, block activation of Cdks. This is important to allow cells to undergo DNA repair before entering mitosis. When mitosis is initiated, dephosphorylation of Cdc25 and dissociation from 14-3-3 allows Cdc25 to dephosphorylate Cdks in the nucleus and hence allows cells to enter mitosis.

**1.10.1 14-3-3 during the G1/S phase of the cell cycle upon DNA damage**

When the cells receive DNA damage stimuli, the ATM/ATR pathway is activated. ATM and ATR are kinases belonging to the PI3K family and their activation is required for G2 arrest (Cliby *et al*, 1998; Rudolph & Latt, 1989). ATM/ATR activates downstream Chk1/Chk2 kinase, and mitogen-activated kinase p38 activation (Nyberg *et al*, 2002; Bao *et al*, 2001; Bulavin *et al*, 2001; Forrest & Gabrielli, 2001; Giles *et al*, 2003). In G1/S phase, Chk1 is phosphorylated at S296 and is located diffusely in the nucleoplasm, and it interacts with 14-3-3 $\gamma$  (Kasahara *et al*, 2010). Association between Chk1 and Cdc25A is bridged by 14-3-3 $\gamma$  (Kasahara *et al*, 2010) and this association results in phosphorylation of Cdc25A by Chk1 at S76 (Sanchez *et al*, 1997; Jin *et al*, 2003). Phosphorylation of Cdc25A also confers association with 14-3-3, which subsequently sequester Cdc25A in the cytoplasm (Chen *et al*, 2003), leading to the degradation of Cdc25A (Jin *et al*, 2003; Kang *et al*, 2008) in a proteasome-dependent manner (Kasahara *et al*, 2010). As a consequence, Cdc25A cannot associate with Cdk2 (Peng *et al*, 1997). On the other hand, 14-3-3 $\sigma$  also has a role in this phase of the checkpoint, where it associates directly with the Cdk2 and Cdk4, and thereby prevents cell cycle entry by inhibiting cyclin-Cdk activity (Laronga *et al*, 2000). All of these studies showed that different 14-3-3 isoforms may play distinct roles in regulating cell cycle through targeting different components of the cell cycle.

14-3-3 has also been reported to have a role in regulating cell cycle through p27. p27 associates with importin- $\alpha$ , which is a protein involved in

transporting proteins across the nuclear envelope into the nucleus (Goldfarb *et al.*, 2004). Since Cdk1s are nuclear proteins, localisation of p27 in the nucleus is crucial for it to inhibit Cdk1s (Slingerland & Pagano, 2000; Cheng *et al.*, 1999) and induce G1 arrest. Like other proteins, 14-3-3 associates with p27 depending on its phospho-status at T157, T179, T197 and T198. AKT can phosphorylate p27 at T157 and T198, with phosphorylation at T157 conferring interaction with 14-3-3  $\beta$ ,  $\epsilon$ ,  $\gamma$ ,  $\zeta$  and  $\theta$ , whilst phosphorylation of p27 at T198 confers interaction with 14-3-3  $\epsilon$ ,  $\eta$  and  $\tau$ , but not  $\beta$  and  $\zeta$  (Shin *et al.* 2002; Kossatz *et al.* 2006; Viglietto *et al.* 2002; Fujita *et al.* 2002; Sekimoto *et al.* 2004; Short *et al.* 2010). This association between 14-3-3 and p27 perturbs p27 interaction with importin- $\alpha$ , since the importin- $\alpha$  interaction site overlaps with 14-3-3 site at T157 (Sekimoto *et al.*, 2004). In this way, 14-3-3 keeps p27 away from importin- $\alpha$  and it remains in the cytosol (Sekimoto *et al.*, 2004).

#### 1.10.2 14-3-3 in G2/M of cell cycle upon DNA damage stimuli

At the G2/M transition in the cell cycle, 14-3-3 proteins are also involved to ensure that the cell undergoes proper checkpoint regulation before entering mitosis. At this phase of the cell cycle, Cdc25B and Cdc25C, which act upstream of the Cdk1/Cyclin B complex (Boutros *et al.*, 2007; Hoffmann *et al.*, 1993; Baldin *et al.*, 1997; Lammer *et al.*, 1998), as well as p53, are regulated by 14-3-3. Following DNA damage response, Chk1/Chk2 is activated, leading to phosphorylation of Cdc25B (Forrest & Gabrielli, 2001; Giles *et al.*, 2003; Uchida *et al.*, 2004a; Astuti *et al.*, 2010) and Cdc25C (Peng



*et al*, 1997; Esmenjaud-Mailhat *et al*, 2007; Chan *et al*, 2011), which then confers interaction with 14-3-3, sequestering Cdc25 into the cytosol, leaving Cdk1/Cyclin B complex in an inactive state (Uchida *et al*, 2004b; Dalal *et al*, 2004; Esmenjaud-Mailhat *et al*, 2007).

p53 can be phosphorylated at multiple sites following DNA damage. Phosphorylation of p53 by ATM/ATR kinases at its N-terminus confers interaction with 14-3-3 $\sigma$  or  $\tau$  (Saito *et al*, 2002; Seong & Ha, 2012), which protects it from Mdm2-mediated degradation and nuclear export, thereby increasing p53 stability and transcriptional activity (Craig *et al*, 1999; Teufel *et al*, 2009; Yang *et al*, 2003; Rajagopalan *et al*, 2010). Chk1/Chk2 also can phosphorylate p53 (Ou *et al*, 2005) at S315, S366, S376, S378, and T387, which are located within the region containing putative NES signals at its C-terminus (Shaulsky *et al*, 1990). Interestingly, phosphorylation of p53 around this region has an impact on p53 activity, particularly its DNA binding ability (Hupp *et al*, 1992; Halazonetis *et al*, 1993; Waterman *et al*, 1995). Phosphorylation on S315 stimulates p53 transactivation and tumour suppressive function (Slee *et al*, 2010; Blaydes *et al*, 2001), but whether phosphorylation of this residue confers interaction with 14-3-3 is yet to be known. When both S376 and S378 are phosphorylated under non-irradiated condition, p53 does not associate with 14-3-3 (Waterman *et al*, 1998). However, following exposure to DNA damage, S376 of p53 is dephosphorylated, while S378 and T387 are phosphorylated by Chk1/2, and this creates a consensus binding site for 14-3-3 $\gamma$ ,  $\epsilon$ ,  $\theta$  and  $\sigma$  proteins and allows association with p53. This in turn increases the affinity of p53

binding to DNA and subsequent activation of p53 responsive promoters (Agarwal *et al*, 1995; Waterman *et al*, 1998). Following this p53 activation, the expression of its downstream target p21 is increased (Agarwal *et al*, 1995), leading to inhibition of nuclear Cdk kinase activity and thus causing a cell cycle arrest (Harper *et al*, 1995; Hermeking *et al*, 1997; Rahman *et al*, 2014; James *et al*, 2014).

### 1.11 14-3-3 and cancer

Considering the number of critical cellular control pathways that can be regulated by different 14-3-3 isoforms, it is not surprising that perturbation in their levels of expression have been implicated in the development of many human cancers. Among the different 14-3-3 isoforms, 14-3-3 $\sigma$  which is also known as human epithelial marker-1 (HEM-1) or stratifin has been implicated directly in cancer development. As described before, it is closely linked to the regulation of p53 tumour suppressive function and stability, and 14-3-3 $\sigma$  levels are downregulated by promoter methylation in multiple malignancies (Iwata *et al*, 2000; Suzuki *et al*, 2000; Moreira *et al*, 2004; Yatabe *et al*, 2002; Logsdon *et al*, 2003; Ferguson *et al*, 2000), although not in cervical cancers (Holm *et al*, 2009; Sano *et al*, 2004). Whilst 14-3-3 $\sigma$  seems to exert a tumour suppressive role, the other 14-3-3 isoforms have potentially pro-oncogenic activities. For example, many isoforms can interact in a phosphorylation-dependent manner with ETS variant 1 (ETV1). This is a DNA-binding transcription factor which regulates matrix-metalloproteinase-1 and -7 (MMP1 and MMP7) expression. Interaction with

14-3-3 increases ETV1 transcription activation of these MMPs, and thereby contributes to cancer invasion (Oh *et al*, 2013). Likewise, downregulation of 14-3-3 $\zeta$  and 14-3-3 $\sigma$  using small interfering RNA and overexpression showed that 14-3-3 $\sigma$  is required for TGF- $\beta$ 1-mediated growth inhibition whereas 14-3-3 $\zeta$  negatively modulates such growth inhibitory responses (Hong *et al*, 2010). In addition, 14-3-3 $\zeta$  maps to human chromosome 8q23 which happens to be a region frequently amplified in metastatic cancers (Ghadimi *et al*, 2003; Tada *et al*, 2000). Indeed, the levels of 14-3-3 $\zeta$  are increased in different tumours including pancreatic (Shen *et al*, 2004), stomach (Jang *et al*, 2004), oral squamous cell carcinoma (Arora *et al*, 2005; Matta *et al*, 2007), lung (Fan *et al*, 2007) and breast cancers (Green & Streuli, 2004). Upregulation of 14-3-3 $\zeta$  has been implicated in epithelial-to-mesenchymal transition (Keshamouni *et al*, 2006; Huang *et al*, 2013), indicating its significant role in tumourigenesis and metastasis. Recent proteomic studies suggested that 14-3-3 $\zeta$  could play a role in linking and manipulating multiple cellular events in cervical cancers (Higareda-Almaraz *et al*, 2013). Overexpression of  $\zeta$  contributes to anchorage independent growth, whilst downregulation of  $\zeta$  reduces tumour growth (Neal *et al*, 2009). In mammary epithelial cells, overexpression of  $\zeta$  increases AKT activity, which in turn, phosphorylates Mdm2 and translocates Mdm2 into the nucleus, leading to p53 degradation (Danes *et al*, 2008). 14-3-3 $\zeta$  also forms a complex with  $\beta$ 1-integrin, which subsequently mediates integrin-induced Rac-1 activation, thus initiating motility-inducing pathways and increased cell migration (O'Toole *et al*, 2011). Moreover, a recent study shows that in non-small cell lung cancer, 14-3-3 $\zeta$  forms a complex with heat

shock protein 27 (Hsp27), and the overexpression of this complex contributes to cell invasion and metastasis, which is also correlated with poor prognosis (Zhao *et al*, 2014).

Other 14-3-3 isoforms may also play important roles in carcinogenesis. Recent studies suggested that besides 14-3-3 $\zeta$ ,  $\epsilon$  and  $\beta$  isoforms are also contributing to proliferation, survival, migration, metastasis of cancer cells (Bai *et al*, 2014; Okayama *et al*, 2014) and also contribute to chemotherapy resistancy (Hodgkinson *et al*, 2012b, 2012a). Due to the increasing data indicating roles for 14-3-3 in carcinogenesis, they have been suggested as cancer biomarkers (Neal & Yu, 2010; Hodgkinson *et al*, 2012b; Padden *et al*, 2014). As mentioned earlier, although 14-3-3 predominantly localises in the cytoplasm, it is also expressed in the nucleus, and this cytoplasmic-nuclear localisation may be used as a biomarker for prognosis of cancer. For example, the nuclear pool of  $\epsilon$  may serve as a tumour suppressor, as decreased nuclear expression of  $\epsilon$  correlates with poor prognosis of colorectal cancer patients (Wang *et al*, 2012), whilst upregulation of 14-3-3 $\epsilon$  in the cytoplasm leads to downregulation of E-cadherin and subsequently contributes to epithelial-mesenchymal transition (Liu *et al*, 2013).

### **AIM OF THIS STUDY**

All of the above indicates that the different 14-3-3 isoforms have different potentials to contribute towards human cancer progression in different anatomical settings. To date, there is limited information on the role of E6 post-translational modification in regulating E6 interactions with proteins other than PDZ domain-containing substrates, and there is no information on whether phospho-E6 might associate with other classes of cellular proteins. Considering the above discussion, 14-3-3 proteins are prime candidates. Since 14-3-3 regulates many cellular processes, and can be considered to be a group of "hub" proteins, E6 interaction with them might offer the virus a way to manipulate multiple cellular regulating pathways, these include affecting cell cycle regulation, DNA damage response, p53 transcriptional transactivation, apoptosis and carcinogenesis. Therefore the aim of this study is to investigate the propensities of different HR HPV E6 oncoproteins to be regulated by different kinases and to understand the functional relevant of such post-translational modification for E6 function.

## CHAPTER 2: MATERIALS AND METHODS

### 2.1 Cell culture and transfection

HeLa, HEK293, HaCaT and H1299 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin-streptomycin (100 U/ml), and glutamine (300 µg/ml). Cells were cultured in an incubator at 37°C with 5% CO<sub>2</sub>.

HeLa cells were transfected with siRNA against the designated genes using Lipofectamine RNAiMax transfection reagent (Invitrogen). HEK293 cells were transfected using calcium phosphate precipitation (Wigler *et al*, 1979), whilst transient transfection of HaCaT and H1299 cells was performed using polyethylenimine (PEI) (Boussif *et al*, 1995).

### 2.2 Plasmids.

In order to generate glutathione *S*-transferase (GST) fusion proteins, HPV-11, HPV-16, HPV-18 E6 and HPV-31 E6 proteins, hDlg and Scribble were cloned and expressed in pGEX2T as described previously (Thomas *et al*, 1996a; Gardiol *et al*, 1999; Pim *et al*, 2000; Nagasaka *et al*, 2010). The GST HPV-31 and -58 E6 fusion proteins were generated by subcloning PCR amplified pcDNA:HPV-31E6 and -58E6, respectively, into compatible *Bam*H I and *Eco*R I restriction sites of pGEX2T. The pGEX2T:HPV-18 E6 mutants R153A, T156E, T156D, S82A and S82A/T156E, as well as -31E6delPDZ and 31E6 T145AdelPDZ fusion proteins were produced using

the Gene Tailor Mutagenesis kit (Invitrogen). The pcDNA:HA- HPV-18E6 R153A was generated by subcloning pGEX2T:HPV-18E6 R153A into compatible *Bam*H I and *Eco*R I restriction site of pcDNA. The GST HPV-33E6 was done by direct subcloning from pcDNA:HPV-33E6 into compatible *Bam*H I and *Eco*R I restriction sites of pGEX2T. The GST-HPV 51 E6 was generated by mutational alteration of the HPV-18E6 PBM to match the PBM of HPV-51, and the PCR amplified product was then subcloned into compatible *Bam*H I and *Eco*R I restriction sites of pGEX2T.

Primer sequences used are:

Type of E6		Primer sequences
18	R153A (F):	(F) 5'- AACGACTCCAACGAGCGAGAGAA ACACAAGTA-3',
		(R) 5'-TCGTTGGAGTAGTTCCTGTCGTGC-3',
	T156D	(F): 5'-CTCCAACGACGCAGAGAAGACCAA GTATAAG-3'
		(R): 5'-TTCTCTGCGTCGTTGGAGTCGTTCC-3'
	T156E	(F): 5'-CAACGACGCAGAGAAGAACAAGTAT AAGAATTC-3'
		(R): 5'-TTCTCTGCGTCGTTGGAGTCGTTCC-3'
	S82A	(F): 5' - AGAGAATTAAGACATTATGCAGACT CTGTGTA - 3'
		(R): 5' - ATAATGTCTTAATTTCTAATTCTA GAA-3'
33		(F): 5'- AGGGATCCATGTTTCAAGACACTG AGG - 3'
		(R): 5' - CCGAATTCTCAACAGTGCAGTTTCT CTAC - 3'
18:51		(F): 5' - TTTGGATCCATGGCGCGCTTTGAGG ATCCA - 3'
		(R): 5' - GAATTCTACTTGCGTTTCATTGCG - 3'
58		(F): 5' - ATGGATCCATGTTCCAGGACGCA GAG - 3'
		(R): 5' - CGGAATTCTTACACTTGTGTTTGT CTGC - 3'
31	delPDZ	(F): 5' - CCGGATCATGTTTCATCAAAAATC - 3'
		(R): 5' - CCGAATTCTTAAGTACGAGGT - 3'
	T145AdelPDZ	(F): 5' - CCGGATCATGTTTCATCAAAAATC - 3'

		(R): 5' - CCGAATTCTTATGCACGAGTC - 3'
	S82A	(F): 5' - CGATGGTATAGATATGCAGTGTATG GAACAAC - 3'
		(R): 5' - GTTGTTCCATACACTGCATATCTATA CCATCG - 3'

Resulting plasmids were all verified by DNA sequencing (Eurofins MWG Operons). These HPV-18 and -31 E6 mutants are all depicted schematically in Figure 11. The pCA:HA-HPV-18E6 R153A was obtained by subcloning the HPV-18 E6 R153A excised from pGEX2T:HPV-18E6 R153A using *BamH* I and *Xba* I, the DNA was then ligated into the pCA vector digested with the compatible restriction enzymes (Tomaić *et al*, 2009a). All the expression constructs were transformed into *E. coli* strain DH5- $\alpha$ .

The pGWI:HA-tagged hDlg and pGWI:HA-tagged MAGI-1 expression plasmids have been described previously (Gardiol *et al*, 1999; Glaunsinger *et al*, 2000). The pSCM:Flag-14-3-3 $\zeta$  and  $\sigma$  expression plasmids were kindly provided by Prof. Haian Fu; pGWI:HA- HPV-18 and -16E6 were kindly provided by Ron Javier; the Flag-p53 expression plasmid was kindly provided by Georgine Faulkner.

### 2.3 GST fusion protein production and purification.

GST and GST-tagged fusion proteins (GST-18 E6, -16 E6, -11 E6, -31 E6, -18E6 R153A, -18 E6 T156E, -18 E6 T156D, -18 E6 S82A and -18 E6 S82A/T156E) were expressed and purified as described previously (Thomas *et al*, 1996b). In brief, the clones harbouring the appropriate expression plasmids, transformed into *E. coli* strain DH5- $\alpha$  were grown in 40ml of



Luria Broth (LB) culture media containing 75mg/ml Ampicillin overnight at 37°C. The bacterial cultures were then transferred into 400ml of LB culture media containing 75mg/ml Ampicillin and incubated at 37°C for 1 hour (hr). Recombinant protein expression was then induced by addition of isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG, Sigma) to a final concentration of 1 nM and incubated for a further 3 hrs. For low producing clones, such as pGEX:HPV-31 E6 and its mutants, pGEX:HPV-33E6 and pGEX:HPV-18:51E6, the induction was carried out at room temperature (RT) overnight. After centrifugation at 4225x g for 5 minutes, the bacterial pellets were lysed with 10ml of 1X PBS containing 1% Triton X-100, and sonicated twice for 30 seconds (sec). The lysates were then centrifuged again at 7648x g for 10 minutes. The supernatants were collected and incubated with glutathione-conjugated agarose resin on a rotating wheel overnight at 4°C. The resins were centrifuged briefly at 1075x g for 3 minutes and the supernatant was discarded. The resins were then washed thrice with 1X PBS containing 0.3% Triton X-100. The resins were then mixed with glycerol to 10% v/v and kept at -20°C for long term storage.

#### **2.4 *In vitro* phosphorylation.**

For each phosphorylation 2 to 5 $\mu$ g of GST fusion proteins were washed with 1X phosphate-buffered saline (PBS) containing 0.1% Triton X-100, followed by washing twice with the respective kinase buffers. The buffers used are as follows:

Kinase	Content of kinase buffer
PKA	25 mM Tris HCl, pH 7.5, 10mM MgCl <sub>2</sub> and 70mM NaCl
p21-Activated kinase (PAK)	50mM Hepes, pH 7.4, 12.5mM NaCl, 1.5mM MgCl <sub>2</sub> , 0.5% Tween 20, 1.5mM MnCl <sub>2</sub>
AKT	25 mM Tris HCl, pH 7.5, 10mM MgCl <sub>2</sub> and 5mM DTT
CamK II	Kinase was diluted in 1X NEBuffer (New England BioLabs) for Protein Kinases (PK) supplemented with 200μM ATP, 1.2μM calmodulin and 2mM CaCl <sub>2</sub> .

*In vitro* phosphorylation of the fusion proteins was carried out using 20μl kinase buffer containing 2.5μCi <sup>32</sup>γ-ATP and 25 Units (U) of cAMP-Dependent Protein Kinase, Catalytic Subunit (Promega), or 250U activated CamK II (NEB), or 0.2μg of AKT1 and 4μl of Reaction Buffer A from AKT1 Kinase enzyme system (Promega) 30°C for 45 minutes. When assessing susceptibility to a specific kinase, the whole reaction mix was analysed directly by SDS-PAGE and autoradiography.

## 2.5 *In vitro* phosphorylation and binding assays.

Purified GST fusion proteins (pre- and post-phosphorylation with cold ATP) were incubated with *in vitro* translated and radiolabelled 14-3-3ζ, hDIg and MAGI-1 for 1 hr at room temperature. Proteins were translated *in vitro* using a Promega TNT kit and radiolabeled with [<sup>35</sup>S]cysteine or [<sup>35</sup>S]methionine (Perkin Elmer). Equal amounts of *in vitro* translated proteins were added to GST fusion proteins bound to glutathione agarose (Sigma) and incubated for 1 hr at RT on a rotating wheel. After washing

thrice with 1X PBS containing 0.1% Triton X-100, the bound proteins were subjected to SDS-PAGE and analysed by autoradiography.

For direct binding assays, I used purified recombinant His-tagged 14-3-3 isoforms: 14-3-3 $\zeta$ ,  $\eta$ ,  $\gamma$  (from Abcam),  $\epsilon$ ,  $\beta$  (from EnzoLife),  $\sigma$  and  $\tau$  (from Geneway). The purified GST fusion proteins (pre- and post-phosphorylation with cold ATP) were incubated with 10ng each of the purified recombinant 14-3-3 isoforms at 4°C for 1 hr. Complexed proteins were then washed thrice with PBS 0.1% Triton X-100. Bound proteins were subjected to SDS-PAGE and western blotting for detection of His-tagged 14-3-3.

## **2.6 *In vivo* phosphorylation and co-immunoprecipitation.**

HEK293 cells ( $7 \times 10^5$  cells) were seeded onto 10cm<sup>2</sup> dishes and transfected with 10 $\mu$ g of HA-tagged HPV-16 E6, -18 E6, -18 E6 T156E, -18 E6 R153A and -18 E6  $\Delta$ PBM respectively, using calcium phosphate precipitation. Five hours post-transfection, cells were treated with 10 $\mu$ M Forskolin (Fsk) (Calbiochem) for 24 hrs, or with 100nM H89 and 10 $\mu$ M MK2206 for 2 hrs in serum-free media. Cells were collected and lysed using high salt E1A buffer (500mM NaCl, 0.1% NP40 and 50mM Hepes pH 7.0) in the presence of protease inhibitors and phosphatase inhibitors followed by gentle syringing and incubation on ice for 20 minutes. Cell lysate was then centrifuged at 16,100x g for 10 minutes. The extracted lysates were either pretreated with 2000U of  $\lambda$  phosphatase (NEB) at 30°C for 15 minutes or incubated directly with 30 $\mu$ l of monoclonal anti-HA conjugated agarose

beads (Sigma) at 4°C for 3 hrs. Samples were washed thrice with high salt E1A buffer and then subjected to western blot analysis.

To determine the interacting partners of HPV-18 and -16 E6 at different phases of the cell cycle, HA-tagged 18 and -16 E6 were transfected into 293 cells using calcium phosphate precipitation. After 24 hrs, the cells were synchronised using Aphidicolin or Nocodazole as described. Prior to extraction, cells were treated with 50µM of the proteasome inhibitor CBZ (Sigma) for 3 hrs. The cells were then extracted using high salt E1A buffer and co-IP was performed as described above.

## **2.7 Downregulation of endogenous 14-3-3ζ, HPV-18 E6/E7 and cell fractionation analysis.**

Approximately  $1.2 \times 10^5$  HeLa cells were seeded onto 60 mm<sup>2</sup> dishes. To study the effect of 14-3-3 on HPV-18 E6 stability, HeLa cells were transiently transfected with siRNA against 14-3-3ζ (Human YWHAZ ON-TARGET plus SMARTpool, Dharmacon) using Lipofectamine RNAiMax transfection reagent (Invitrogen). HPV 18 E6 and E7 were ablated by transfecting siRNA against HPV 18 E6/E7 (Dharmacon) using RNAi Max (Invitrogen). In all cases, siRNA against luciferase was used as a negative control.

For cell fractionation, approximately  $2.4 \times 10^5$  of HaCaT cells were seeded onto 60 mm<sup>2</sup> dishes and were transiently transfected with plasmids

expressing HA-18 E6 and HA-18E6  $\Delta$ PBM using PEI. Cells were collected by trypsinisation 72 hrs post transfection. Samples were then subjected to cell fractionation analysis, where samples were divided into cytoplasmic and nuclear fractions using the NE-PER fractionation kit (Pierce) as described in the manufacturer's protocol. Protein extracts were quantified by Bradford Assay and analysed by western blotting.

## **2.8 Half-life experiments.**

At 24 hrs post-transfection, cells were treated with cycloheximide (50 $\mu$ g/ml in dimethyl sulfoxide [DMSO]) to block protein synthesis at different time points. DMSO alone-treated cells were used as the control. Cells were then lysed with high salt E1A buffer and samples were analysed by western blotting for E6, and the intensity of the bands on the X-ray film was quantified using the ImageJ program. The standard deviation was calculated from at least three independent assays.

## **2.9 Mass spectrometry analysis**

The GST-31 E6 fusion protein was phosphorylated *in vitro* by PKA as described above. The protein was then washed twice with 1X PBS containing 0.1% Triton X-100, and followed by washing using PKA buffer. The sample was then subjected to mass spectrometry by Dr. Michael Myers as follows:

For sample preparation, the proteins were eluted from the beads using 50ng of sequencing grade trypsin (Promega) in 20mM diammonium phosphate pH 8.0 for 6 hrs at 37°C. The supernatant was then removed from the beads. The cysteine residues were reduced and alkylated by boiling for 2 minutes in the presence of 10mM Tris (2-carboxyethyl) phosphine (Pierce, Milan, Italy) followed by incubating with 20mM acetaminophen (Sigma) for 1 hr at 37°C. The reactions were stopped by adding 0.1% of acetic acid. The mixture was desalted using C18 Ziptips (Millipore, Milan, Italy) and lyophilised.

The desalted samples were then injected onto the nanobore column in buffer A (10% methanol/0.1% formic acid). The column were constructed using Picofrit columns (NewObjective, Woburn, MA, USA) packed with 15 cm of 1.8 mm Zorbax XDB C18 particles using a homemade high-pressure column loader and the column was developed with a discontinuous gradient and sprayed directly into the orifice of an LTQ ion trap mass spectrometer (Thermo Electron, San Jose, CA, USA). A cycle of one full scan (400 - 1700 m/z) followed by eight data-dependent MS/MS scans at 25% normalized collision energy was performed throughout the LC separation. RAW files from the LTQ were converted to mzXML files by READW (version 1.6) and searched against the Ensembl human protein database and the NCBI nr Viral database using the Global Proteasome Machine interfaced to the X-Tandem algorithm (version 2006.06.01.2)

### 2.10 *In vivo* degradation assays

For *in vivo* degradation assay, HEK293 cells were transiently transfected with plasmids expressing Flag-tagged 14-3-3 $\zeta$  in the presence or absence of HA-tagged HPV-18 E6, at a ratio of 3:1. As transfection efficiency controls, the cells were also co-transfected with LacZ vector control. As a positive control, the cells were also transfected with plasmid expressing HA-tagged hDlg, in the presence or absence of HA-tagged HPV-18 E6 plasmids. After 24 hrs, the cells were treated with H89 or Fsk as described before. The samples were analysed by SDS-PAGE and the levels of proteins were ascertained by western blotting.

### 2.11 Cell synchronisation and FACS analysis

HEK293 cells were transiently transfected with plasmids expressing HA-tagged HPV-18 or -16 E6 as described above. After 24 hrs, the cells were treated with 5 $\mu$ g/ml of Aphidicolin (Sigma) for another 24 hrs to synchronise cells in G1/S phase. Synchronised G1/S phase cells were also released from Aphidicolin treatment for 5 hrs to obtain a population of cells in S phase. To synchronise cells in the G2/M phase of the cell cycle, cells were treated with 125 $\mu$ g/ml of Nocodazole for 18 hrs. The cells were extracted using high salt E1A buffer and HA-tagged E6 proteins were immunoprecipitated as described above.

HeLa cells ( $3.5 \times 10^5$  cells) were seeded on 60mm<sup>2</sup> dishes. After 24 hrs, cells were treated with 5 $\mu$ g/ml of Aphidicolin (Sigma) for another 24 hrs to

synchronise cells in G1/S phase. Synchronised G1/S phase cells were also released from Aphidicolin treatment for 5 hrs to obtain a population of cells in S phase. To synchronise cells in the G2/M phase of the cell cycle, cells were treated with 125µg/ml of Nocodazole for 18 hrs.

Alternatively, the cells were treated with 2.5mM thymidine for 16 hrs. The cells were washed twice with 1X PBS and replenished with fresh culture media. After 9 hrs, thymidine was added to further synchronise the cells for additional 16 hrs. The cells were then collected at 0 hrs (G1/S), 5 hrs (S phase) and 9 hrs (G2/M). Cells in the different cell cycle phases were extracted and analysed for E6 expression, with the cell cycle phases verified using propidium iodide staining and FACS analysis using a FACScalibur Cell Sorter (Becton Dickinson).

## **2.12 Co-immunoprecipitation of p53 and luciferase reporter assays**

H1299 cells ( $7 \times 10^5$  cells) were seeded onto 10cm<sup>2</sup> dishes and transfected with 10µg of plasmids expressing HA-tagged HPV-18 and -18 E6 ΔPBM, respectively, using calcium phosphate precipitation, and 5 hrs post-transfection the cells were either left untreated or treated with Nocodazole as described above for 18 hrs. Three hours prior to harvesting, cells were treated with CBZ as described above. Cells were collected and lysed with high salt E1A buffer. The samples were subjected to co-immunoprecipitation, as described above, using anti-Flag conjugated agarose beads (Sigma). HeLa cells ( $7 \times 10^5$  cells) were also seeded onto



10cm<sup>2</sup> dishes and cells were either transiently transfected with siRNA against HPV-18 E6/E7, or were treated with CBZ, or Nocodazole as before, or left untreated. The cells were collected and extracted using high salt E1A buffer. The cell lysate was then incubated with monoclonal anti-p53 (DO-1) antibody at 4°C overnight at a dilution of 1:200. The lysate was then further incubated with Sepharose A beads to immunoprecipitate endogenous p53 and the bound proteins. The samples were then subjected to SDS-PAGE and western blotting.

To analyse p53-mediated p21 promoter transcription activity, luciferase reporter assays were performed by transiently transfecting p21-Luciferase reporter plasmid with pCMV-Renilla plasmid alone as negative control, and with plasmid expressing Flag-tagged p53 as positive control, or co-transfected with plasmids expressing HA-tagged HPV-18E6, and its mutants  $\Delta$ PBM and R153A into H1299 cells using PEI. After 24 hrs, the cells were either left untreated or treated for 3 hrs with CBZ. The cells were then collected and luciferase assays were performed using the Dual-Luciferase Reporter System (Promega), as recommended by the manufacturer's protocol. In brief, the cells were lysed using 1X Passive lysis buffer (Promega) for 15 minutes at RT. Lysate was then centrifuged briefly and 5 $\mu$ l of the supernatant was then added to 20 $\mu$ l of Luciferase assay reagent. 20 $\mu$ l of Stop and Glow reagent was then added and the p21 promoter transcriptional activity was measured using TD-20/20 Luminometer (Turner Designs).

### 2.13 Antibodies and western blotting.

Immunoprecipitation samples or total cell extracts were obtained by lysing the cells directly in 2X SDS-PAGE sample buffer. Western blotting and processing was then done as described previously (Massimi *et al*, 2008). Briefly, nitrocellulose was blocked in 5% milk in 1X PBS/0.5% Tween20 (PBST) for 1 hr at 37°C. Primary antibodies were diluted (1:1000) in 1X PBST and were incubated at RT for 1 hr with gentle rocking. After washing thrice with 1X PBST, the blot was incubated for 1 hr at RT with the appropriate secondary antibodies conjugated horseradish peroxidase (HRP, Dako), diluted (1:2000) in 1X PBST/5% milk. After extensive washing with 1X PBST, the blot was developed using the ECL detection system (GE Healthcare) according to the manufacturer's protocol. Any modifications to this protocol are detailed below with the description of the antibody concerned.

The following antibodies were used according to the protocol outlined above: mouse anti-6X His (BD), mouse anti-HA (Roche), mouse anti-p84 (Abcam), mouse anti-ubiquitin protein ligase 3A (E6AP) (BD Bioscience), rabbit anti- $\alpha$ -actinin, mouse anti-p53 DO-1, rabbit anti-14-3-3 $\zeta$ ,  $\epsilon$ ,  $\gamma$  and  $\theta/\tau$ , goat anti-14-3-3 $\sigma$  and mouse anti-14-3-3 $\beta$ , mouse anti-SAP97 and mouse anti- $\alpha$ -tubulin (all from Santa Cruz) and appropriate secondary antibodies conjugated to horseradish peroxidase (HRP; Dako).

Mouse monoclonal antibody against HPV-18 E6 (1:1,000; MAb399), directed against the HPV-18 E6 N-terminus, was generated and generously provided by the ArborVita Corporation. Western blots using this antibody

were performed as above, with the following modifications: the blocking buffer was 1X Tris-buffered saline (TBS)/0.1% Tween20 (1X TBST) with 5% milk and 5% BSA; MAb399 was used at a dilution of 1:1000 for 2 hrs at RT. The anti-phospho-E6 ( $\alpha$ -pE6) specific antibody (custom-made by Eurogentec) was generated using  $\text{H}_2\text{N-RQERLQRRRET}(\text{PO}_3\text{H}_2)\text{QV-COOH}$  peptide in rabbits and was used in western blots with the following modifications: the blocking buffer was 1X TBST with 3% BSA;  $\alpha$ -pE6 was used at a dilution of 1:500 for overnight at 4°C. The blots were washed with 1X TBST.

## CHAPTER 3: RESULTS

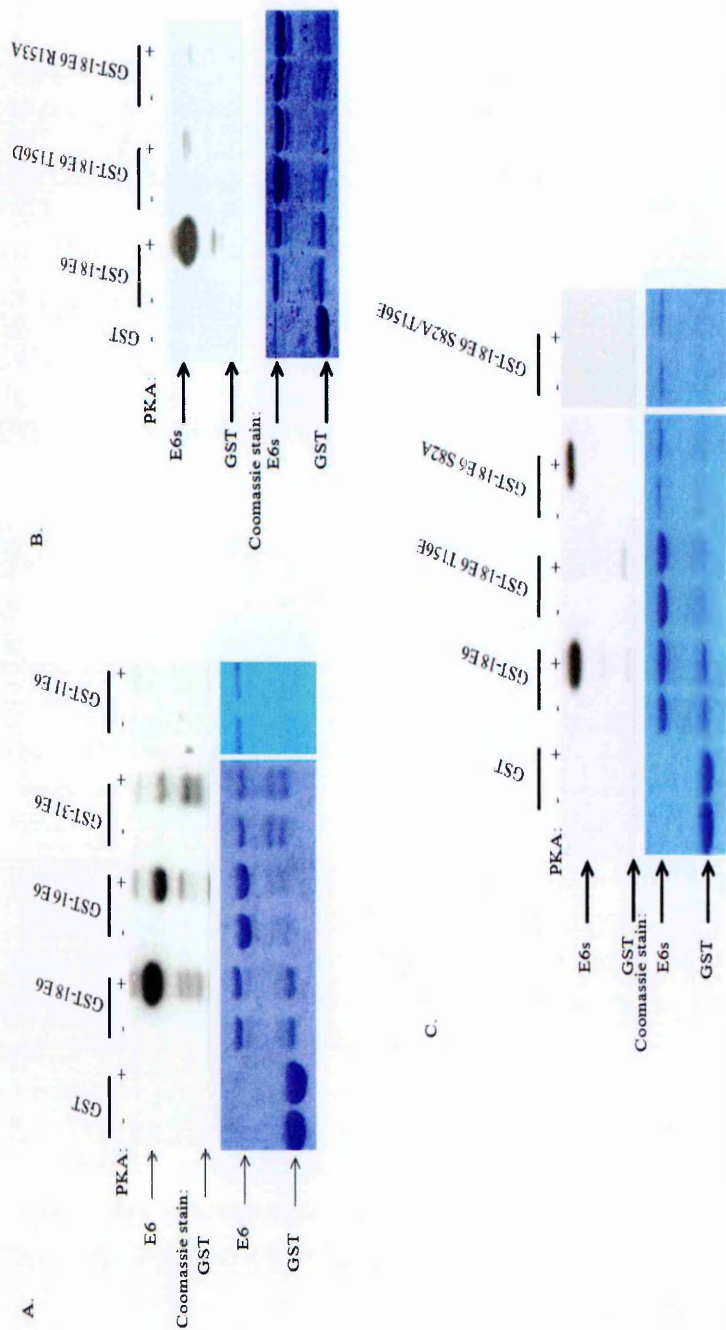
### 3.1 High risk HPV E6 oncoproteins are common substrates of PKA.

Previous studies have shown that HPV-18 E6 and carboxy terminal peptides derived from several other HR HPV E6 oncoproteins are substrates of PKA (Kühne *et al*, 2000). As mentioned before, the PBM is conserved among the HR HPV E6 proteins although, as can be seen from Figure 10, there is a degree of sequence variation between the different HR HPV E6 proteins. I was therefore first interested in determining whether these different E6 proteins were all similar substrates of PKA. To investigate this, the relative susceptibilities of HR HPV-16, -18 and -31 E6 to phosphorylation by PKA were investigated. The LR-11 E6, which lacks a PKA consensus phospho-acceptor site, was also included to serve as a negative control. The different E6 proteins were expressed as GST fusion proteins and purified. These were then incubated with purified PKA and radiolabeled ATP; after 20 minutes the levels of phosphorylation were ascertained by SDS-PAGE and autoradiography. The results obtained in Figure 11(A) demonstrate that HPV-16, HPV-18 and HPV-31 E6 are all substrates of PKA, whilst HPV-11 E6 is not, in agreement with previous studies (Kühne *et al*, 2000). However, these results also demonstrate that among these, HPV-18 E6 is the strongest substrate, followed by HPV-16 E6, with HPV-31 E6 being a much weaker PKA substrate.

It has been shown previously that the major phospho-acceptor site on HPV-18 E6 is the Thr156 (Kühne *et al*, 2000), however phospho-site predictions,

<b>AKT consensus site</b>		<b>RXRXXS/T</b>
<b>PKA consensus site</b>		<b>RXXT/S</b>
<b>14-3-3 consensus</b>		<b>RRXS/T</b>
<b>PDZ binding motif</b>		<b>XS/TXV</b>
	<b>82</b>	<b>156</b>
18 E6	....RHYSDS.....	RRRETQV*
18 E6 T156E	....RHYSDS.....	RRREEQV*
18 E6 T156D	....RHYSDS.....	RRREDQV*
18 E6 R153A	....RHYSDS.....	R4RETQV*
18 E6 S82A	....RHYADS.....	RRRETQV*
18 E6 S82A/T156E	....RHYADS.....	RRREEQV*
16		GRCMSCCRSSRTRRETQL*
33		....RCAACWRSRRRETAL*
51		.....RTRQRNETQV*
58		....RCAVCWRPRRRQTQV*
31		.....RCIVCWRRPRTETQV*
31 del PDZ		.....RCIVCWRRPRT*
31 T145Adel PDZ		.....RCIVCWRRPRA*

**Figure 10. Amino acid sequence of the HR HPV E6 PDZ binding motif (PBM).** The alignments show the PBMs of HPV-18, -16, -31, -33, -51 and -58. Also shown are the different mutant derivatives, HPV-18 E6 T156E, T156D and R153A, together with the HPV-18 E6 S82A mutation and the S82A/T156E double mutant. The HPV-31 E6 mutations delPDZ and T145AdelPDZ are also shown. For comparison, the consensus recognition sequence for AKT (Obata *et al*, 2000), PKA (Pearson & Kemp, 1991), 14-3-3 (Muslin *et al*, 1996) and the PDZ binding motif (Songyang *et al*, 1997) are shown.



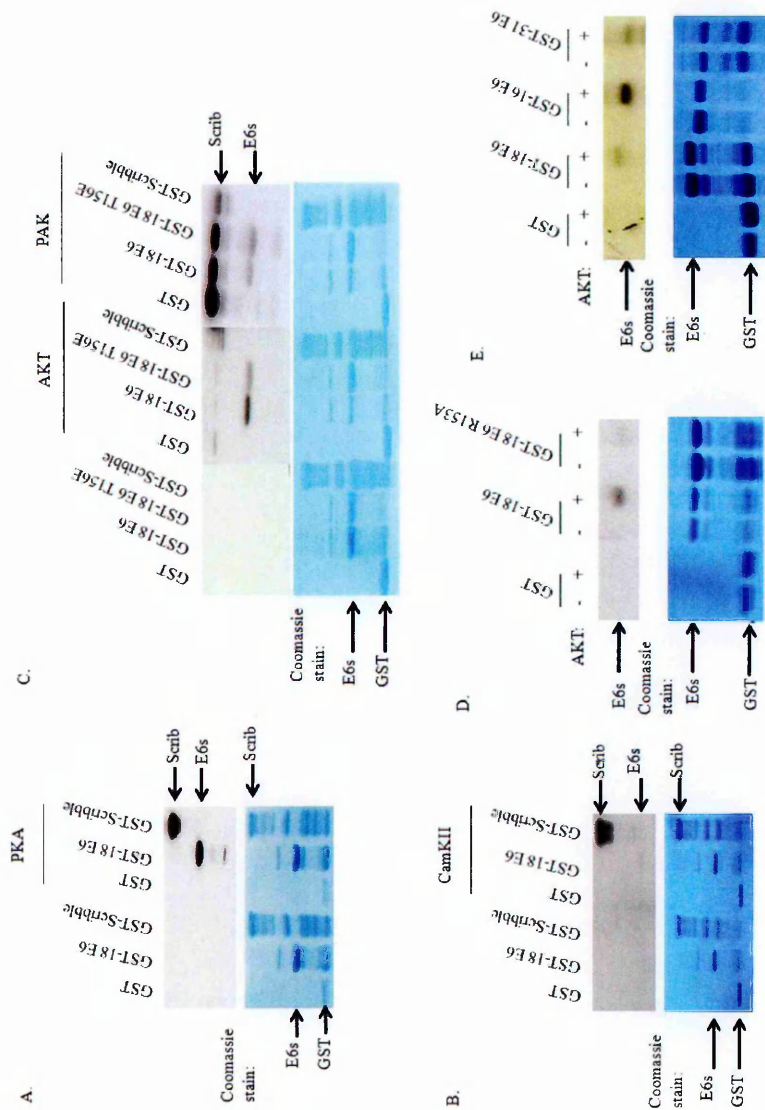
**Figure 11. High risk HPV E6 oncoproteins are phosphorylated by PKA to differing degrees.** A. The purified HPV-18, -16, -31 and -11 E6 GST fusion proteins were either untreated (-) or incubated with PKA and  $^{32}\text{P}$ - $\gamma$ -ATP (+) as indicated. Proteins were then subjected to SDS-PAGE and autoradiography. The upper panel shows the autoradiogram and the lower panel shows the Coomassie stain of the gel. B and C. PKA phosphorylation of different HPV-18 E6 mutant GST fusion proteins as indicated. In each case the upper panel shows the autoradiogram and the lower panel shows the Coomassie stained gels. Arrows indicate the relevant GST proteins.

as shown in Appendix, suggest that S82 might also be recognized by PKA. To verify whether Thr156 is indeed the major site of PKA phosphorylation of HPV-18 E6, a series of HPV-18 E6 mutants were generated. These included 2 phospho-mimic mutants, T156D and T156E, and the R153A mutation which destroys the PKA consensus recognition site. Mutations were also introduced at the predicted phospho-acceptor site, both singly with S82A and the double mutation of S82A/T156E. All of these mutations are also depicted in Figure 10. In order to assess whether these mutants could be phosphorylated by PKA, they were again expressed as GST fusion proteins, purified and incubated with PKA and radiolabelled  $^{32}\text{P}$ - $\gamma\text{ATP}$ . The results demonstrated that T156 is the only PKA phospho-acceptor site on HPV-18 E6 (Figure 11B and C), with mutations at this residue and at R153, which is essential element of the PKA recognition sequence, destroying PKA phosphorylation of the E6 protein, whilst mutation of S82A (Figure 11C) has no major impact upon the susceptibility of E6 to phosphorylation by PKA.

The above results demonstrated quite surprising differences in the susceptibility of HPV-18, HPV-16 and HPV-31 E6 proteins to phosphorylation by PKA, despite their all having very similar carboxy terminal residues. Therefore, there is a possibility that other kinases might potentially phosphorylate the different E6 oncoproteins. To investigate this possibility, phosphorylation was performed using kinase predicted p21-activated Kinase (PAK) (personal communication with Dr. Sally Roberts) and those scored highly in the phospho-site prediction software (Obenauer *et al*, 2003):  $\text{Ca}^{2+}$ /Calmodulin kinase II (CamKII) and Protein kinase B (PKB)

or AKT (shown in Appendix). The susceptibility of HPV-18 E6 to PKA phosphorylation was used as a control, and this was compared with Scribble, a protein known to be phosphorylated by multiple kinases (Li *et al*, 2011b; Nagasaka *et al*, 2010; Yoshihara *et al*, 2011; Skouloudaki *et al*, 2009). The results again showed a high levels of HPV-18 E6 phosphorylation by PKA (Figure 12A), with little or no phosphorylation by CamK II (Figure 12B). There is very weak phosphorylation of E6 by PAK, which is not T156-dependent (Figure 12C). However, there is a significant level of phosphorylation by AKT (Figure 12D) and the level of phosphorylation of HPV-18 E6 T156E by AKT is reduced (Figure 12C), indicating that AKT phosphorylation also occurs within the PBM. To further determine whether this was mediated by recognition of the carboxy terminal consensus site (Figure 10), the assay was also repeated using the R153A mutant and, as can be seen from Figure 12D, this also abolishes phosphorylation by AKT. Having found that AKT was the kinase most likely to phosphorylate E6 in addition to PKA, HPV-16 and -31 E6 were then analysed for their susceptibility to be phosphorylated by AKT. The results in Figure 12E demonstrate that HPV-16 E6 is the better substrate for AKT, with HPV-31 E6 being phosphorylated more weakly, at a level similar to that seen with HPV-18 E6. Taken together these results demonstrate that HR HPV E6 proteins can be substrates for either PKA or AKT, albeit with different efficiencies depending upon the HPV types. In the case of HPV-18 E6, the phospho-acceptor site for both AKT and PKA is T156, and both kinases recognise the same consensus site as defined by the R153 residue.



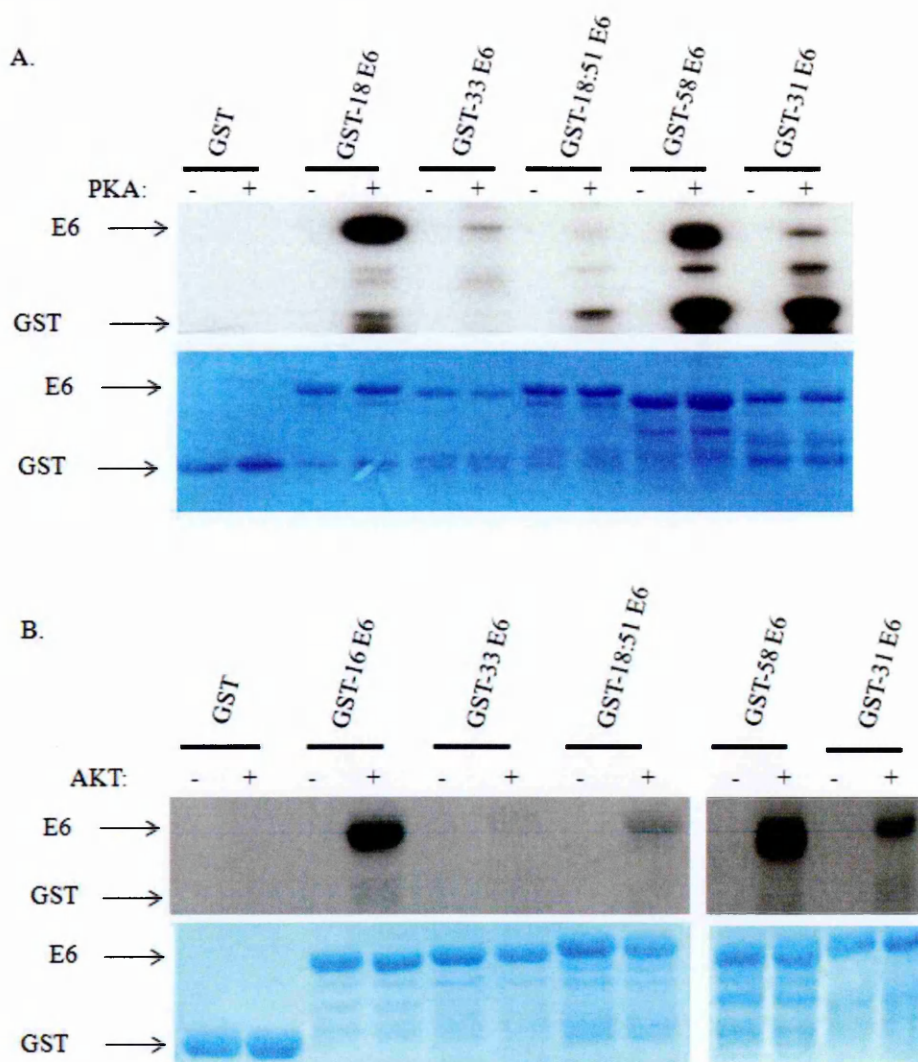


**Figure 12. Comparative analysis of E6 phosphorylation by different kinases.** A-C. Phosphorylation of GST fusion proteins of HPV-18 E6, the HPV-18E6 T156E mutant, and Scribble with PKA, CamK II, AKT and PAK as indicated. The upper panels show the autoradiograms and the lower panels show the Coomassie stained gels. D and E. The comparative phosphorylation of HPV-18, HPV-16, HPV-31 E6 and the HPV-18 E6 R153A mutant GST fusion proteins by AKT as indicated. The upper panels show the autoradiograms and the lower panel shows the Coomassie stained gels. Arrows indicate the relevant GST fusion proteins.

### **3.2 Phosphorylation of the E6 PBM is conserved across multiple HPV types.**

Having shown significant variation in the degree to which different HR HPV E6 proteins are phosphorylated, I next wanted to extend the analysis to additional HPV types. To do this, HPV-33, -18:51 and -58 E6 proteins were expressed as GST fusion proteins, with HPV-16, -18 and -31 E6 GST fusion proteins for comparison. These HPV types were chosen as they were subtly different within their last 6 amino acids in their C-terminal from HPV-16, -18 and -31 E6 (Refer to Figure 10). HPV-18:51 E6 GST fusion protein used in this study was generated by mutating C-terminal PBM of HPV-18 E6 to mimic that of HPV-51 E6. The purified proteins were incubated with purified PKA or AKT, together with radiolabeled ATP. The levels of E6 phosphorylation were then ascertained by SDS-PAGE and autoradiography. The results in Figure 13A demonstrate that HPV-18 E6 is the strongest substrate for phosphorylation by PKA, being closely followed by HPV-58 E6. HPV-33, -18:51 and -31 E6 are all somewhat weaker substrates for PKA. In the case of AKT, HPV-16 E6 and -58 E6 are the strongest substrates. HPV-31 then follows, with HPV-33 and -18:51 being poor substrates for AKT (Figure 13B). These results demonstrate that, whilst all the high-risk HPV E6 proteins analysed can be phosphorylated by PKA and/or AKT, and perhaps other kinases which remain to be identified, there are major differences in the efficiency with which this occurs.

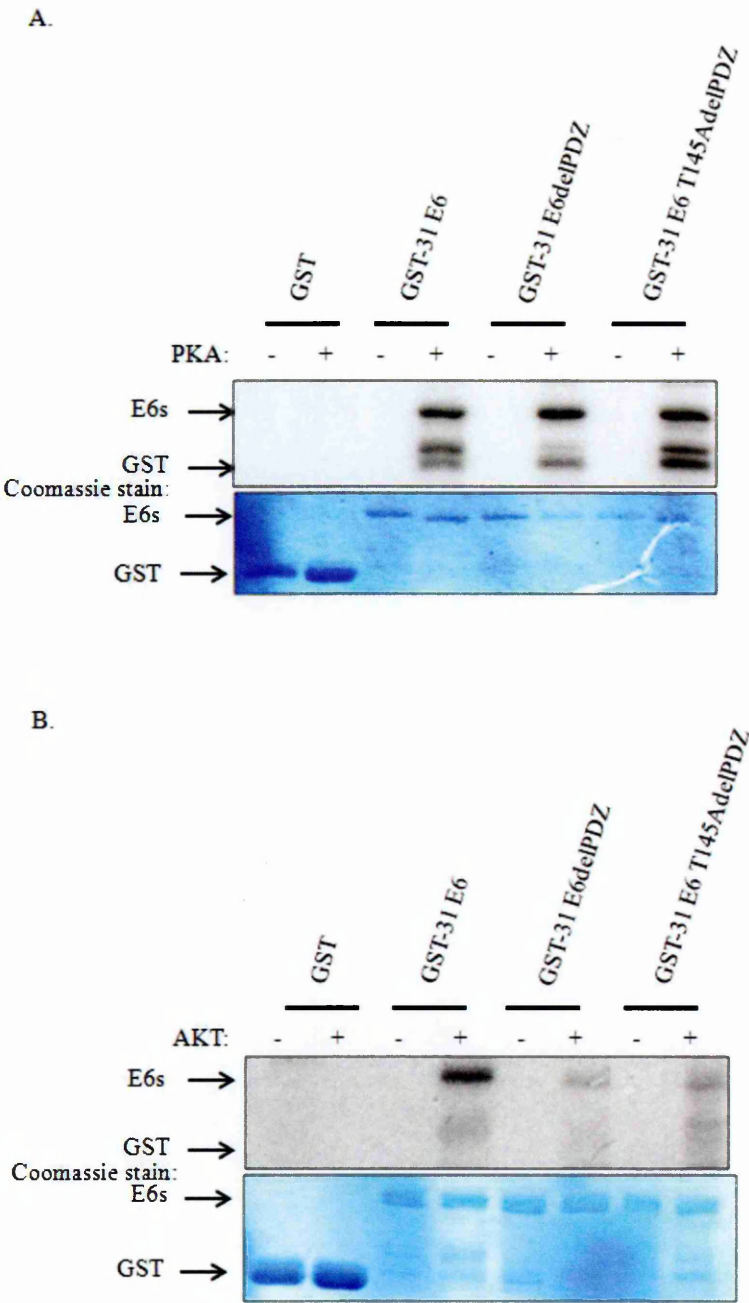
Analysis of the HPV-31 E6 carboxy terminal sequence (Figure 10) indicates the existence of two potential phospho-acceptor sites (Threonine residues)



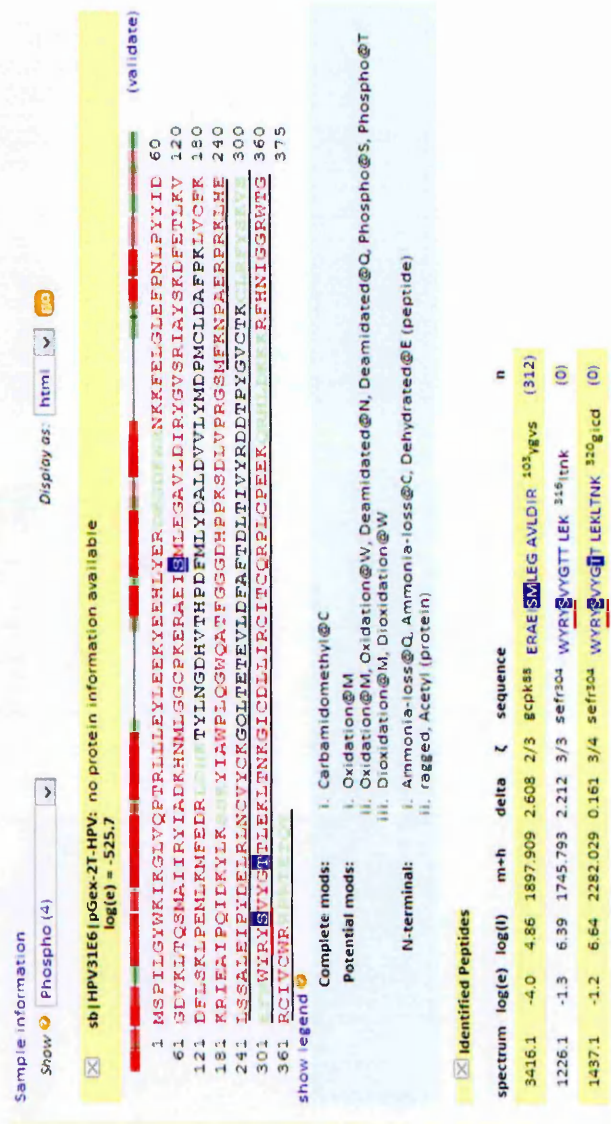
**Figure 13. High risk HPV E6 oncoproteins are phosphorylated by PKA or AKT.** A. The purified GST fusion proteins of HPV-18, -33, -18:51, -58 and -31 E6, either untreated (-) or incubated with PKA (+) and  $^{32}\text{P}$ - $\gamma$ ATP as indicated. Proteins were analysed by SDS-PAGE and autoradiography. B. HPV-16, -33, -18:51, -58 and -31 E6 GST fusion proteins, either untreated (-) or treated with AKT (+) and  $^{32}\text{P}$ - $\gamma$ ATP as indicated. In each case the upper panels show the autoradiograms and the lower panels show the Coomassie stained gels. Arrows indicate the relevant GST proteins.

within this region: one within the PBM (T147) and one located just upstream (T145). Since HPV-31 has been used extensively as a model virus for analysing the HPV life cycle and its role in the development of malignancy (Frattoni *et al*, 1996; Moody & Laimins, 2009), I was interested in investigating whether HPV-31 E6 was indeed phosphorylated within the PBM. To identify which of these potential sites was phosphorylated, two mutations within the HPV-31 E6 carboxy terminus (see Figure 10) were generated. The purified GST fusion proteins were then subjected to phosphorylation with PKA and AKT and the results obtained are shown in Figure 14. As can be seen, removal of the PBM and mutation of T145A in HPV-31 E6 has no effect on the levels of PKA phosphorylation (Figure 14A). In the case of AKT, removal of the PDZ domain almost completely abolished phosphorylation by AKT, and mutation of T145A has no further effect (Figure 14B). This indicates that HPV-31 E6 is phosphorylated by AKT at T147 within the the PBM. In order to identify the PKA phospho-acceptor site, mass spectrometry of the PKA phosphorylated HPV-31 E6 was performed. The result in Figure 14C reveals that the phospho-acceptor site used by PKA is S82, indicating that the potential consequences of PKA phosphorylation of HPV-31 will be very different from those seen with other high risk HPV E6 oncoproteins.

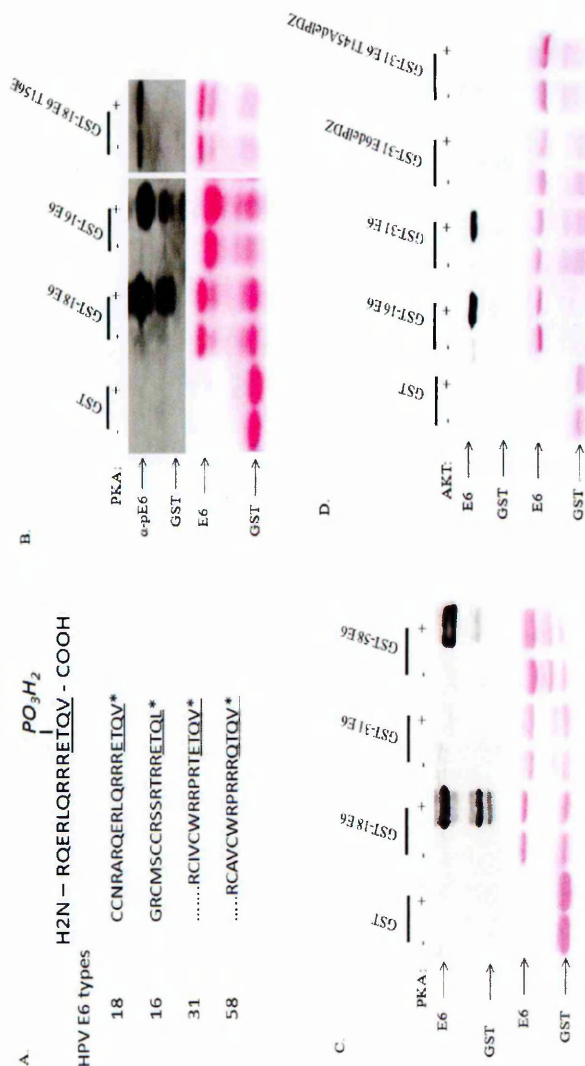
In order to develop tools to be able to assess the phospho-status of HPV E6 *in vivo*, an anti-phospho-E6 specific ( $\alpha$ -pE6) antibody was generated which could specifically recognise phosphorylated forms of HPV-18 E6. This was done through Eurogentec, where rabbits were immunised with a 13-mer C-terminal phospho-peptide of HPV-18 E6 (as shown in Figure 15A). In order







**Figure 14. HPV 31 E6 is differentially phosphorylated by PKA and AKT.** A. The purified GST fusion proteins of HPV-31 E6 wild type and PBM mutants, 31 E6delPDZ and 31 E6 T145AdelPDZ, either untreated (-) or incubated with PKA (+) and <sup>32</sup>P-γATP as indicated. Proteins were analysed by SDS-PAGE and autoradiography. The upper panel shows the autoradiogram and the lower panel shows the Coomassie stain of the gel. B. A similar experiment to Panel A, but GST fusion proteins were either untreated (-) or incubated with AKT (+). C. Mass spectrometry analysis of purified GST-31 E6 phosphorylated by PKA as performed in Panel A. Underlined in black is the amino acid sequence of HPV-31 E6 with residues in red identified in mass spectrometry, residues in green are not covered. Upstream sequence corresponds to GST. The three phospho-containing peptides identified are shown below. One peptide was derived from GST (RAEIS), whilst two were derived from HPV-31 E6 with S82 being the common site identified. Underlined in red is the possible PKA phosphorylation site (RYS), and the phospho-acceptor residue is highlighted in blue.



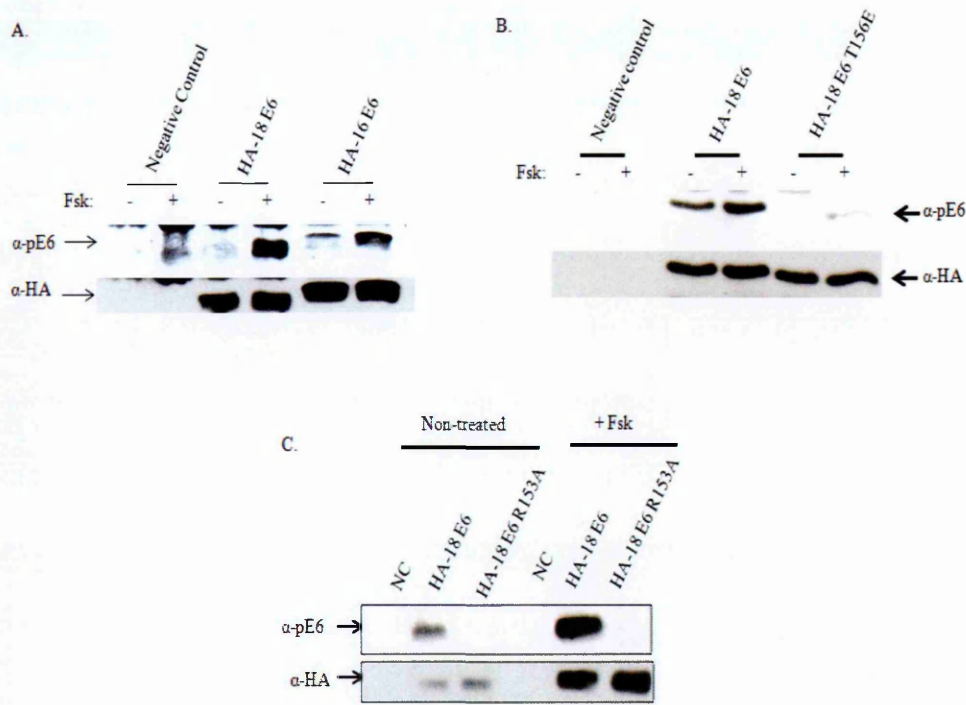
**Figure 15. Detection of differential phosphorylation of HR HPV E6 by western blotting using anti-phospho-E6 specific ( $\alpha$ -pE6) antibody.** **A.** The amino acid sequence of the peptide used to immunise the rabbit in the process of producing the anti-phospho-E6 specific antibody ( $\alpha$ -pE6). Also shown are the amino acid sequence of the C-terminal PBM of the different HR HPV type E6 proteins. **B.** The HPV-18, -16 and phosphomimetic -18 E6T156E GST fusion proteins were either untreated (-) or incubated with PKA (+) in the presence of cold ATP. These were detected by western blotting using anti-phospho-E6 specific antibody. **C.** A similar analysis of GST-HPV-18, -31 and -58 E6 GST fusion proteins, either untreated (-) or incubated with PKA (+) as described in Panel A. **D.** The purified GST- HPV-16, -31, and -31 E6 mutants, -31 E6delPDZ and -31 E6 T145AdelPDZ, either untreated (-) or incubated with AKT (+) as indicated. Arrows indicate the relevant GST proteins. The lower panel shows the Ponceau stain of the nitrocellulose membrane confirming equal levels of protein loading.

to characterise the antibody, western blots were done using GST-16 and -18 E6 pre- and post-phosphorylation with PKA. The result in Figure 15B shows that the  $\alpha$ -pE6 antibody can recognise PKA phosphorylated HPV-16 and -18 E6 and, to a lesser extent, the HPV-18 E6 T156E phospho-mimic mutant. As can be seen in Figure 15B, T156E was recognised by the  $\alpha$ -pE6 antibody in the absence of phosphorylation, but the levels of detection do not change significantly following incubation with PKA. This suggests that the phospho-mimic charge mutation on HPV-18 E6 does behave as such, at least with respect to recognition by the  $\alpha$ -pE6 antibody. I then extended the same analysis to determine whether this  $\alpha$ -pE6 antibody can cross-react with other HPV E6s. The result in Figure 15C shows that the  $\alpha$ -pE6 antibody can recognise HPV-58 E6 phosphorylated by PKA. In contrast, PKA phosphorylated HPV-31 E6 is not detected by this antibody (Figure 15C), consistent with the HPV-31 E6 PKA phospho-acceptor site lying outside the PBM. In contrast, it is clear that wild type HPV-31 E6 phosphorylated by AKT is recognized by the  $\alpha$ -pE6 antibody, whereas the two carboxy terminal mutants of E6 are not (Figure 15D). These results demonstrate that the  $\alpha$ -pE6 antibody raised against HPV-18 E6 can detect multiple HR HPV E6 proteins when these are phosphorylated within the PBM. Furthermore, these results confirm that HPV-18, -16 and -58 E6 are phosphorylated by PKA and AKT within the PBM, whilst HPV-31 E6 can be phosphorylated by both AKT and PKA, although the phospho-acceptor sites are not the same, and only AKT phosphorylates HPV-31 within the PBM.



### 3.3 HPV E6 is phosphorylated by AKT and PKA *in vivo*.

The above results demonstrated that the high risk HPV E6 proteins are potential substrates for PKA, although there is little evidence directly demonstrating E6 phosphorylation within its carboxy terminal region *in vivo*. To investigate this, HEK293 cells were transfected with HA-tagged HPV-16 E6, HPV-18 E6, HPV-18 E6 T156E and HPV-18 E6 R153A expression constructs and incubated in the presence or absence of forskolin (Fsk) in order to stimulate endogenous PKA activity. After 24 hrs the cells were harvested and immunoprecipitations were performed using anti-HA antibody-conjugated agarose beads. The immunoprecipitates were then analysed by western blotting using the anti-HA and  $\alpha$ -pE6 antibodies to detect total and phospho-E6, respectively. The results in Figure 16A, B and C demonstrate a number of interesting features. Firstly, in the absence of Fsk, HPV-18 E6 appears to be only weakly phosphorylated (Figure 16A, B and C), whilst there is a slightly higher level of phosphorylation of HPV-16 E6 (Figure 16A). However, incubation of the cells with Fsk results in a more dramatic increase in the levels of both HPV-18 as compared to HPV-16 E6 phosphorylation as shown in Figure 16A. In contrast, the HPV-18 E6 T156E mutant shows weak reactivity with the  $\alpha$ -pE6 antibody, consistent with the mutation only acting as a partial phospho-mimic (Figure 16B). The PKA mutant, R153A was not readily recognised by  $\alpha$ -pE6 antibody either in the absence or presence of Fsk stimulation (Figure 16C), confirming that this mutation also abolished phosphorylation of the HPV-18 E6 PBM *in vivo*. These results demonstrate that transiently transfected HPV-16 and HPV-18 E6 are highly phosphorylated *in vivo* following stimulation of PKA. In the



**Figure 16. HR HPV E6 is phosphorylated *in vivo*.** **A.** HEK293 cells were transfected with empty vector or the HA-tagged HPV-18 or -16 E6 expression plasmids as indicated in the presence (+) or absence (-) of forskolin (Fsk). After 24 hrs, the cells were harvested and subjected to immunoprecipitation using anti-HA-conjugated agarose beads. The presence of E6 was then detected by western blotting using either the anti-phospho-E6 specific ( $\alpha$ -pE6) antibody to detect phosphorylated E6 (upper panels) or the anti-HA antibody to detect total levels of E6 protein (lower panels). **B.** A similar analysis of HA-tagged HPV-18 E6 or -18 E6 T156E phosphomimic mutant was transiently expressed in HEK293 cells as described in Panel A, the cells were either untreated (-) or incubated with Fsk (+) as indicated. **C.** A similar analysis of HA-tagged HPV-18 E6 or HPV-18 E6 R153A PKA mutant was transiently expressed in HEK293 cells as described in Panel A. The cells were either left non-treated or incubated with Fsk (+Fsk) as indicated.

absence of PKA stimulation the levels of E6 phosphorylation are weak, although minor differences in the levels of phosphorylation exist between the two E6 proteins.

To investigate which kinases were phosphorylating E6 *in vivo*, 293 cells were transfected with HA-tagged HPV-16 and HPV-18 E6 expression plasmids, and after 24hrs the cells were treated with H89 for 2 hrs to inhibit PKA, MK2206 for 2 hrs to inhibit AKT, or Fsk (overnight treatment) to stimulate PKA activity. The cells were extracted and immunoprecipitated using monoclonal anti-HA-conjugated agarose beads. The total and phospho-E6 was detected by western blotting using anti-HA and  $\alpha$ -pE6 antibodies respectively. As can be seen from Figure 17, HPV-18 E6 (Figure 17A) was very weakly phosphorylated in asynchronous culture when compared with HPV-16 E6 (Figure 17B). Inhibition of either AKT or PKA results in a marked decrease in the levels of phosphorylated HPV-16 E6 (Figure 17B), whilst there is a very weak reduction in the levels of phospho-HPV-18 E6 (Figure 17A), although this is difficult to gauge given the low levels of phosphorylation. In addition, stimulation of PKA activity with Fsk induces a dramatic increase in the levels of phospho-HPV-18 E6 (Figure 17A), but only a modest increase in the levels of phospho-HPV-16 E6 (Figure 17B) consistent with the results in Figure 16A. These results suggested that in transiently transfected asynchronously growing cells E6 can be a substrate for both AKT and PKA, but that HPV-16 E6 is phosphorylated to significantly higher levels than HPV-18 E6 under these conditions. However, when PKA activity is elevated, then HPV-18 E6 becomes more heavily phosphorylated and this is consistent with the



**Figure 17. HPV E6s are differentially phosphorylated in asynchronously growing cells.** **A.** HEK293 cells were mock transfected or transiently transfected with the indicated HA-tagged 18 E6 expression plasmids in the presence (+) or absence (-) of H89, forskolin (Fsk) or MK2206 as indicated. After 24hrs the cells were harvested and subjected to immunoprecipitation using anti-HA conjugated agarose beads. The presence of E6 was detected by western blotting using either the anti-phospho-E6 specific ( $\alpha$ -pE6) antibody (upper panels) to detect phosphorylated E6 or the anti-HA antibody to detect total levels of E6 protein. Also shown is the input loading control for  $\alpha$ -actinin. **B.** HEK293 cells were mock transfected or transiently transfected with the indicated HA-tagged 16 E6 expression plasmids in the presence (+) or absence (-) of H89, forskolin (Fsk) or MK2206 as indicated. Immunoprecipitation and western blotting were performed as described in Panel A. **C.** HeLa cells were transfected with control siRNA (siLuc) and siRNA against 18 E6/E7 (siE6/E7). After 72hrs the cells were exposed to forskolin (Fsk) as indicated and then harvested and the levels of phospho-E6 was ascertained by western blotting using anti-phospho-E6 specific antibody. Also shown are the controls of  $\alpha$ -actinin and p53. **D.** HeLa cells were either left untreated or treated with Fsk, H89 or MK2206, or transiently transfected with siE6/E7 as indicated. Total cell extract was analysed by western blotting using  $\alpha$ -pE6 to detect phosphorylated E6. Also included are the controls of  $\alpha$ -actinin and p53.

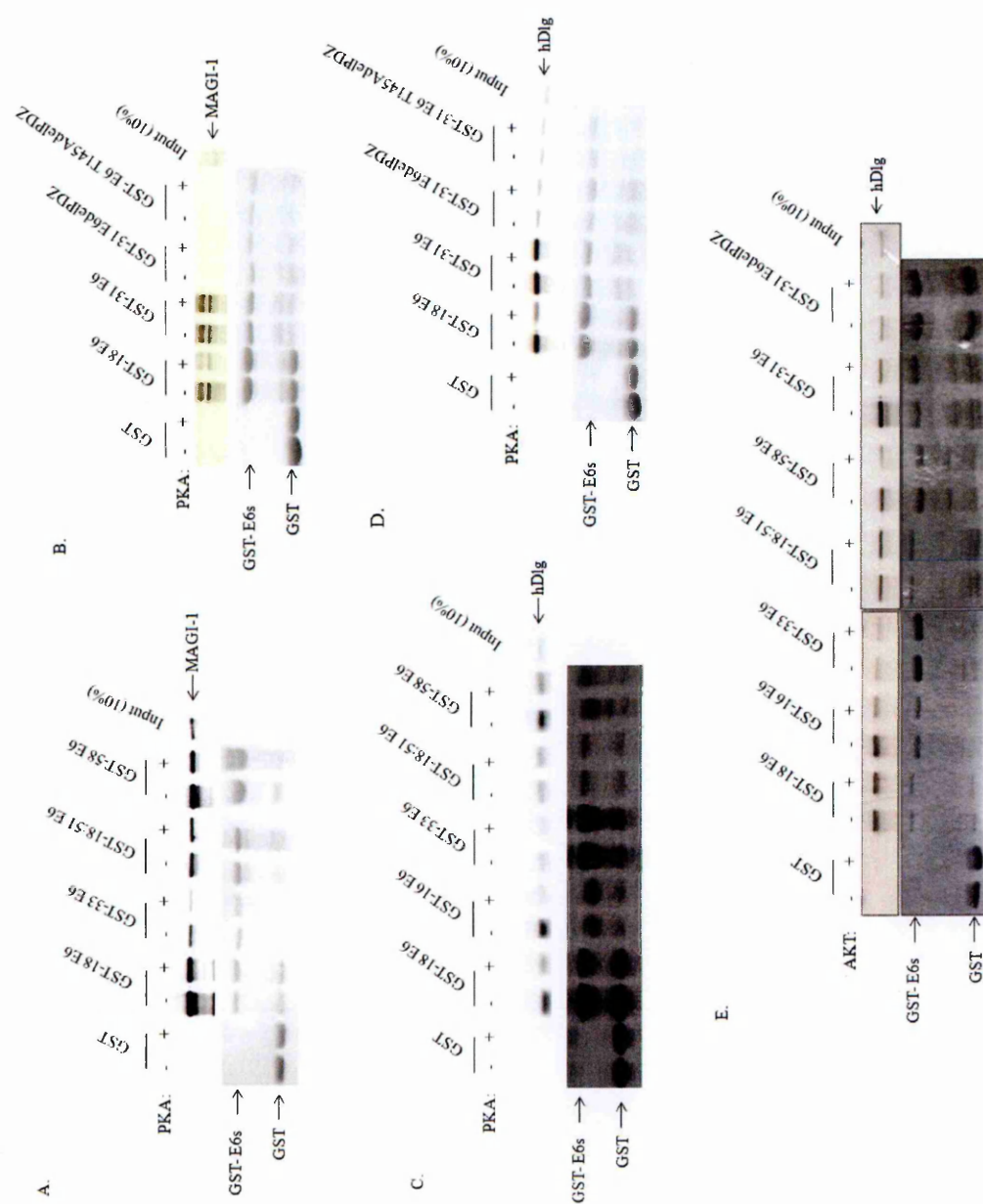
observation of differential phosphorylation of HPV-18 and -16 E6 by PKA and AKT *in vitro*.

I then wanted to ascertain if endogenously expressed E6 is phosphorylated in a similar manner. To do this, HeLa cells were stimulated with Fsk and then analysed by western blotting to determine whether endogenous phospho-E6 can be detected. The results in Figure 17C show low levels of phospho-E6 in unstimulated HeLa cells, but that Fsk treatment induces a dramatic increase in the levels of E6 phosphorylation. The identity of the E6 protein was also verified by performing siRNA E6/E7 ablation of E6 expression. This result shows that the  $\alpha$ -pE6 antibody can recognise the endogenous phospho-E6 in HeLa cells. In order to identify the kinases that phosphorylate HPV-18 E6, HeLa cells were treated with Fsk, H89 or MK2206 and phospho-E6 was detected by SDS-PAGE and western blotting. The result in Figure 17D shows that HPV-18 E6 is phosphorylated at a low level in untreated cells and heavily phosphorylated upon Fsk stimulation. Phosphorylation of HPV-18 E6 was also greatly decreased upon H89 or MK2206 treatment, suggesting that HPV-18 E6 is subject to phosphorylation by both PKA and AKT in HeLa cells. It was also noticed that H89 and MK2206 treatment reduces the levels of p53 in HeLa cells, with a dramatic reduction observed with the use of MK2206. This could be due to the inhibition of kinases that normally phosphorylate p53, as previous studies have shown that phosphorylation of p53 helps maintain its stability (Meek, 1994; Ashcroft *et al*, 1999).

### 3.4 Phosphorylation of E6 negatively regulates interaction with hDlg and MAGI-1.

Previous studies showed that PKA phosphorylation of HPV-18 E6 negatively regulates its interaction with hDlg (Kühne *et al*, 2000), whilst phosphorylation of PBMs is generally assumed to inhibit PDZ recognition (Zhang *et al*, 2007). It was therefore interesting to ascertain whether the other high-risk HPV E6 oncoproteins were subject to a similar pattern of regulation. To do this, a series of *in vitro* pull-down assays were performed using phosphorylated GST-E6s and *in vitro* translated radiolabelled MAGI-1 and hDlg. The results obtained are shown in Figure 18 and demonstrate a number of interesting features. Although there are variations in the degree to which the different E6 proteins bind MAGI-1 and hDlg, with HPV-18 and HPV-58 E6 being the strongest, the phosphorylation of HPV-18, -33, -18:51 and -58 E6 all decreased their ability to interact with MAGI-1 (Figure 18A) and hDlg, except for HPV-18:51 E6 (Figure 18C). Similarly, HPV-16 E6 showed a strong association with hDlg in the absence of phosphorylation, however, a reduction of hDlg recognition by HPV-16 E6 was observed when HPV-16 E6 was phosphorylated by PKA (Figure 18C). Meanwhile, HPV-31 E6 appears to differ: its interaction with MAGI-1 and hDlg is comparable to that of HPV-18 E6, but PKA phosphorylation has no effect on the interaction (Figure 18B and D). This is consistent with the above results showing that HPV-31 E6 is phosphorylated by PKA outside the PBM (Figure 14A and 15C). In both cases the PBM mutation confirmed that HPV-31 E6 PDZ interactions are mediated through classic PBM recognition (Figure 18B and D). These results demonstrate that although the PDZ





**Figure 18. PKA and AKT phosphorylation negatively regulates interaction of HPV E6 with PDZ domain-containing proteins. A and B.** The indicated GST fusion proteins were either untreated (-) or subjected to phosphorylation with PKA (+) in the presence of cold ATP. These were then incubated with *in vitro* translated radiolabelled MAGI-1. Following extensive washing the bound MAGI-1 was detected using SDS-PAGE and autoradiography (shown in the upper panel). The lower panel shows the Coomassie stain of the gel, arrows indicate the relevant proteins. **C and D.** The indicated GST fusion proteins were either untreated (-) or subjected to phosphorylation with PKA (+) in the presence of cold ATP. These were then incubated with radiolabelled *in vitro* translated hDlg as indicated. Following extensive washing, bound proteins were detected using SDS-PAGE and autoradiography shown in the upper panel. The arrows indicate the relevant fusion proteins and *in vitro* translated products. **E.** The indicated GST fusion proteins were either untreated (-) or subjected to phosphorylation with AKT (+) in the presence of cold ATP. These were then incubated with radiolabelled *in vitro* translated hDlg as indicated. Following extensive washing, bound proteins were detected using SDS-PAGE and autoradiography shown in the upper panel. The lower panel shows the Coomassie stain of the gel. The arrows indicate the relevant fusion proteins and *in vitro* translated products.

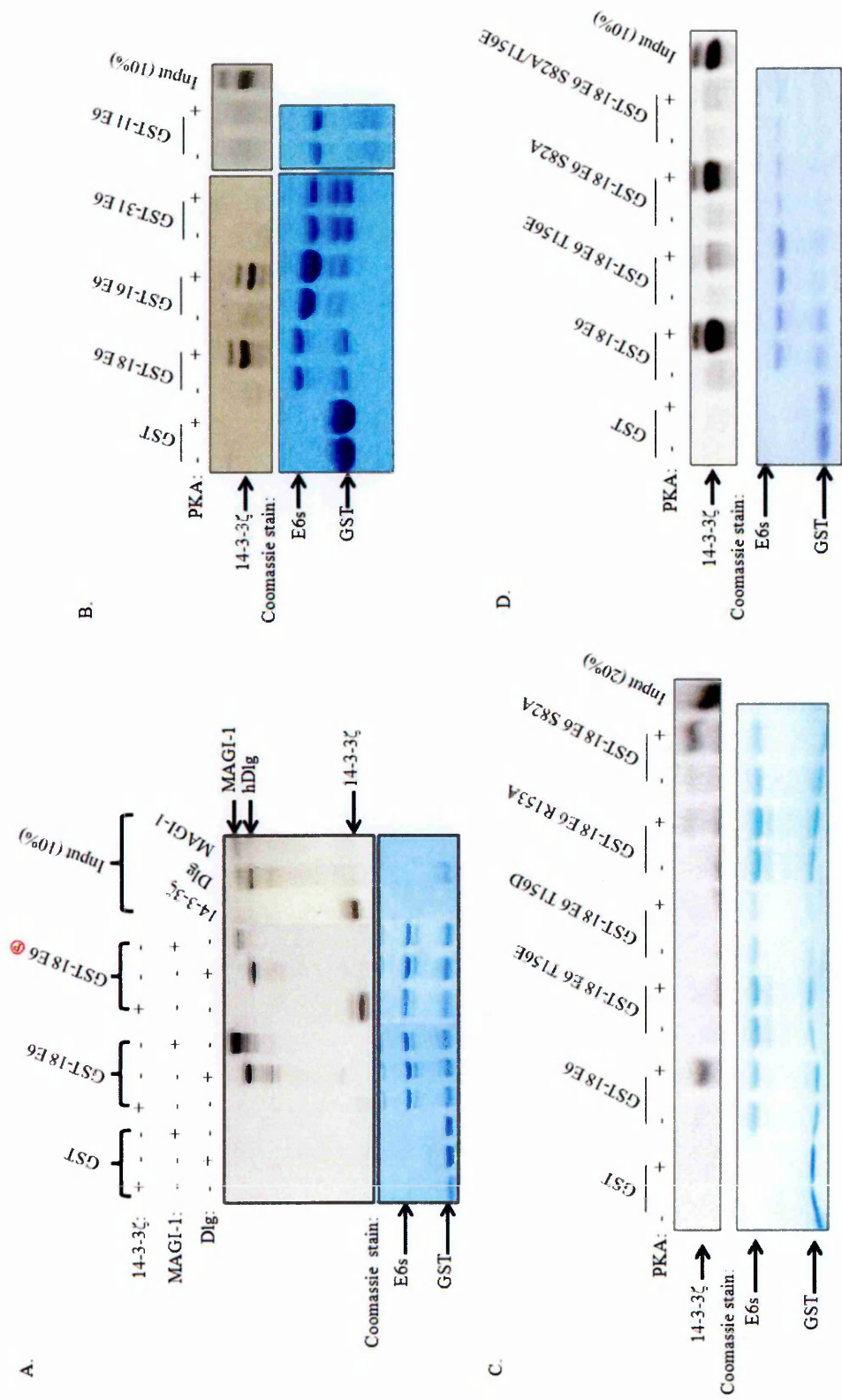


recognition of most HPV E6 oncoproteins is negatively regulated by PKA, this does not hold true for HPV-31 E6.

Since the different HPV E6s display differential patterns of phosphorylation by PKA or AKT, I then wanted to understand whether AKT phosphorylation has a similar effect on the interaction between E6 and PDZ proteins. In order to answer this, a similar *in vitro* phosphorylation of GST-E6 fusion proteins using active AKT was performed, and the effects on interactions with hDlg was analysed. The results shown in Figure 18E reflect the capacity of the different E6 proteins to be phosphorylated by AKT within their PBMs. Thus, HPV-18 and -33 E6 are weak substrates of AKT (as shown in Figure 12E and 13B), and likewise, AKT phosphorylation has no effect on their ability to interact with hDlg. In contrast, HPV-16 and HPV-58 E6 are very good substrates of AKT (as shown in Figure 13B) and this is reflected in the effects on hDlg recognition, where phosphorylation greatly reduces the interaction with the two E6 proteins. As noted above, HPV-31 E6 is phosphorylated by AKT on the T embedded within the PBM (Figure 14B and 15D) and, not surprisingly, phosphorylation of HPV-31 E6 by AKT also results in a strong inhibition of HPV-31 E6 interaction with hDlg. Taken together, these results demonstrate that the capacity of different HPV E6 oncoproteins to be phosphorylated by different cellular kinases varies considerably amongst the different HR HPV E6 oncoproteins, but the consequences of such phosphorylation events within the E6 PBM are always the same, resulting in an inhibition of E6 PBM-PDZ interactions.

### 3.5 Phospho-E6 interacts with 14-3-3 $\zeta$ .

Whilst phosphorylation of HPV E6 inhibits its recognition of PDZ proteins, it is not known if this post-translational modification favors E6 recognition of other cellular proteins. As mentioned before, mass spectrometry analysis performed in our laboratory (personal communication Dr. Vjekoslav Tomać) and performed by Howie *et al.* (2011), indicated that 14-3-3 proteins are potential targets of E6 (Howie *et al.*, 2011), and these are particularly attractive candidates due to their phosphoserine/threonine binding properties. In the mass spectrometry analysis, 14-3-3 $\zeta$  was identified as the one of the E6 target proteins. To investigate potential interactions between 14-3-3 and HPV-18 E6, purified GST-18 E6 fusion proteins were subjected to PKA phosphorylation using non-radiolabelled ATP. Interaction assays were then performed with *in vitro* translated radiolabelled 14-3-3 $\zeta$ . As controls, parallel assays were also performed with *in vitro* translated hDlg and MAGI-1. The results obtained are shown in Figure 19A, and reveal a number of interesting features. In the absence of phosphorylation, 14-3-3 $\zeta$  did not interact with HPV-18 E6, whilst there is strong association of HPV-18 E6 with both hDlg and MAGI-1. However, following phosphorylation of HPV-18 E6 there is a dramatic decrease in the capacity of E6 to recognise both hDlg and MAGI-1 and a corresponding increase in the capacity of E6 to interact with 14-3-3 $\zeta$ . These results demonstrate that the PBM/PKA module is potentially dual functional, depending upon the phospho-status of the T156 residue, and this confers either recognition of PDZ proteins or, alternatively, recognition of 14-3-3 $\zeta$ .



**Figure 19. HPV E6 interacts with 14-3-3 $\zeta$  in a phosphorylation dependent manner.** A. The indicated GST fusion proteins were incubated with radiolabelled *in vitro* translated MAGI-1, hDlg or 14-3-3 $\zeta$ , which were either untreated or subjected to phosphorylation with PKA (+PKA) in the presence of cold ATP as indicated. Following extensive washing, bound proteins were detected using SDS-PAGE and autoradiography (upper panel). The lower panel shows the Coomassie stain of the gel. The arrows indicate the relevant fusion proteins and translated products. **B.** The indicated GST fusion proteins were either untreated or subjected to phosphorylation with PKA (®) in the presence of cold ATP. These were then incubated with radiolabelled *in vitro* translated 14-3-3 $\zeta$ . Following extensive washing, bound proteins were detected using SDS-PAGE and autoradiography (upper panel). The lower panel shows the Coomassie stain of the gel. The arrows indicate the relevant fusion proteins and translated products. **C.** The indicated GST fusion proteins were either untreated (-) or subjected to phosphorylation with PKA (+) in the presence of cold ATP. These were then incubated with *in vitro* translated radiolabelled 14-3-3 $\zeta$ . Following extensive washing the bound 14-3-3 $\zeta$  was detected using SDS-PAGE and autoradiography (upper panel). The lower panel shows the Coomassie stain of the gel. Arrows indicate the relevant proteins. Panels **D.** Assays to monitor 14-3-3 $\zeta$  interaction with the different HPV-18 E6 mutant GST fusion proteins were done as described in Panel C.

Having found that HPV-18 E6 could interact with 14-3-3 $\zeta$ , I extended the studies to determine whether HPV-16 and HPV-31 E6 could also recognise 14-3-3 $\zeta$  in a phosphorylation-dependent manner. To do this, these purified GST-16 and -31 E6 fusion proteins were subjected to phosphorylation by PKA using non-radiolabelled ATP, and binding assays performed with *in vitro* translated radiolabelled 14-3-3 $\zeta$ . The results in Figure 19B again showed a strong interaction between phospho-HPV-18 E6 and 14-3-3 $\zeta$ . A similar phospho-dependent interaction between HPV-16 E6 and 14-3-3 $\zeta$  was also apparent, but HPV-31 E6, which is phosphorylated by PKA outside the PBM, fails to interact with 14-3-3 $\zeta$ . As expected, no interaction was observed between HPV-11 E6 and 14-3-3 $\zeta$ , consistent with the absence of a PBM/PKA module on the carboxy terminus of HPV-11 E6. These results demonstrate that the association between E6 and 14-3-3 $\zeta$  is a reflection of the degree to which the particular E6 oncoprotein can be phosphorylated by PKA in the PBM.

Having shown that phospho-E6 can interact with 14-3-3, I next wanted to investigate the other sequence requirements within the PBM for this interaction to take place. To do this, a panel of HPV-18 E6 mutants (Figure 10) were used. These include the phospho-mimics, T156D and T156E, the potential PKA phospho-acceptor site mutant S82A, the double mutant containing both S82A and the T156E mutation (S82A/T156E), and the PKA consensus recognition mutant R153A. The purified fusion proteins were phosphorylated with PKA and *in vitro* interaction assays were performed by incubating these fusion proteins with *in vitro* translated radiolabelled 14-3-3 $\zeta$  as described above. The results in Figure 19C and D again demonstrate

that phosphorylation of HPV-18 E6 is essential for the interaction with 14-3-3 $\zeta$ , since no association is seen with the R153A mutant. Furthermore, these results also demonstrate little or no influence of the S82 residue upon the ability of E6 to recognize 14-3-3 $\zeta$ . Surprisingly, the two phospho-mimics also failed to interact with 14-3-3 $\zeta$ . This result demonstrates that recognition of 14-3-3 $\zeta$  by HPV-18E6 is strictly dependent upon phosphorylation of T156 and a simple replacement with an acidic residue is not sufficient to confer interaction.

### **3.6 The interaction between phospho-E6 and 14-3-3 $\zeta$ is direct.**

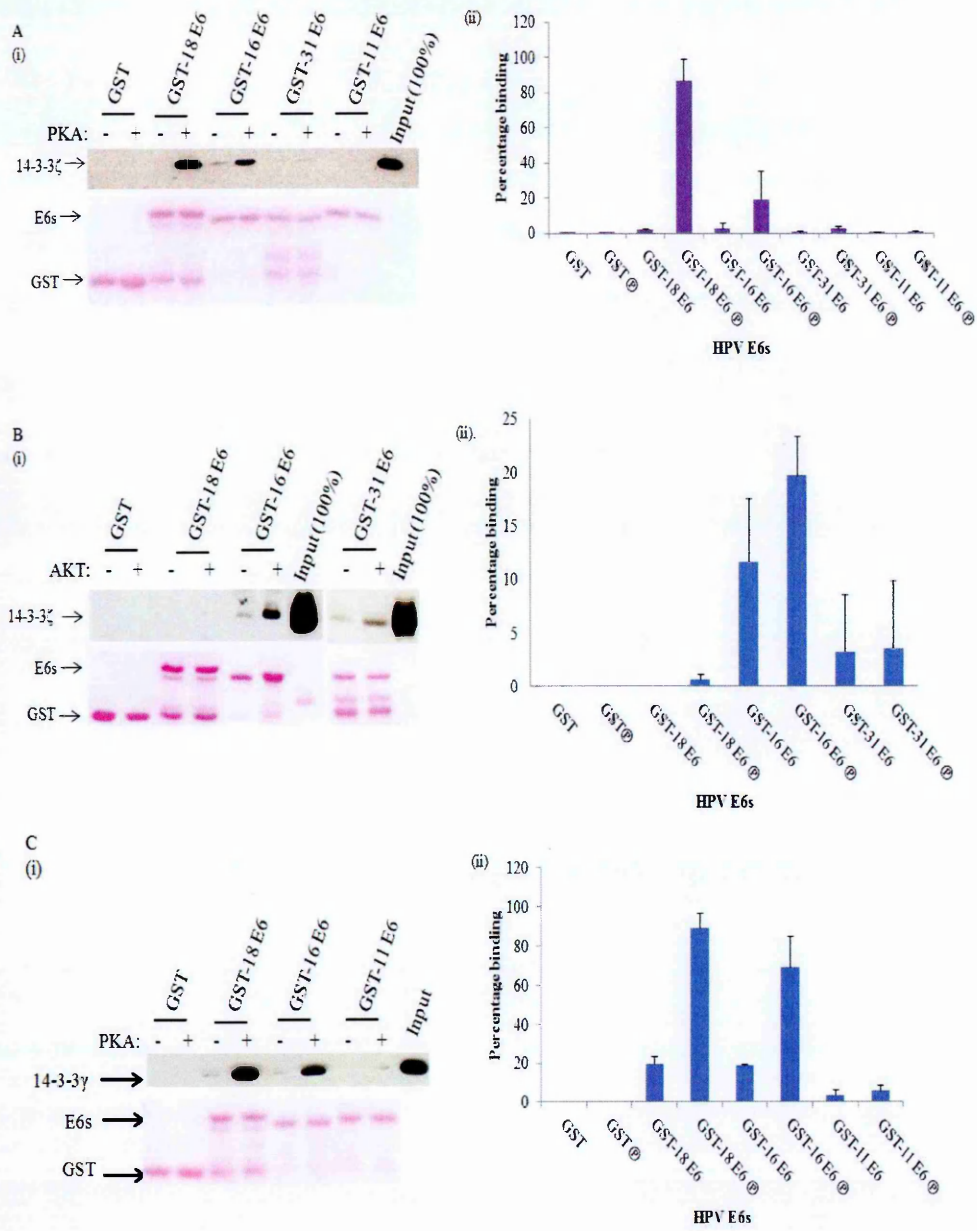
To ensure that the interaction between E6 and 14-3-3 $\zeta$  was direct, and not mediated through an intermediate protein, the interaction assays were repeated using commercially available purified 14-3-3 $\zeta$ . Following PKA phosphorylation of the purified GST-E6 fusion proteins, these were incubated with the purified His-tagged 14-3-3 $\zeta$ , and after extensive washing the bound 14-3-3 $\zeta$  was detected by western blotting using anti-His antibody. The results obtained are shown in Figure 20A (i), with the quantitations in Figure 20A(ii), and these confirm the phospho-specific association between HPV-16 and HPV-18 E6 and 14-3-3 $\zeta$ , with little or no interaction occurring between HPV-31 and HPV-11 E6 and 14-3-3 $\zeta$ , again corresponding to their respective susceptibility to phosphorylation by PKA within the PBM.

Since the above results indicate that HPV-16 E6 and HPV-31 E6 can also be phosphorylated by AKT, the direct binding assays were repeated to investigate whether AKT phosphorylation of these E6 proteins could

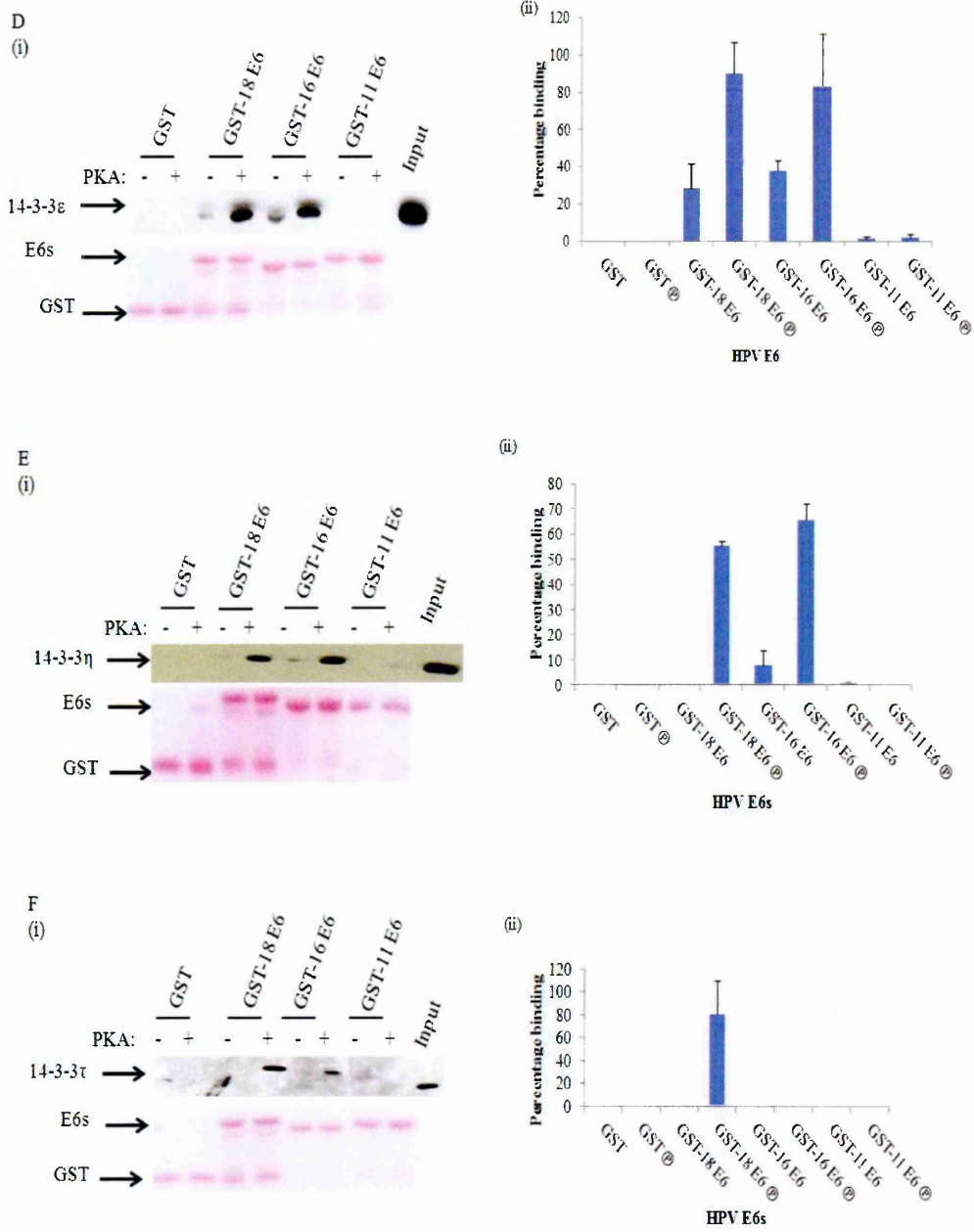
likewise stimulate interaction with 14-3-3 $\zeta$ . The results of this analysis in Figure 20B(i) and 20B(ii) demonstrate that AKT phosphorylation of HPV-16 E6 by AKT does indeed promote interaction with the purified 14-3-3 $\zeta$ . Most interestingly, phosphorylation of HPV-31 E6 by AKT, which in contrast to PKA phosphorylation occurs within the PBM, also confers interaction with 14-3-3 $\zeta$ . This result demonstrates that the capacity of high risk E6 oncoproteins to interact with 14-3-3 $\zeta$  is highly conserved across different HPV types, and that differences are mainly determined by whether phosphorylation occurs in the PBM or not.

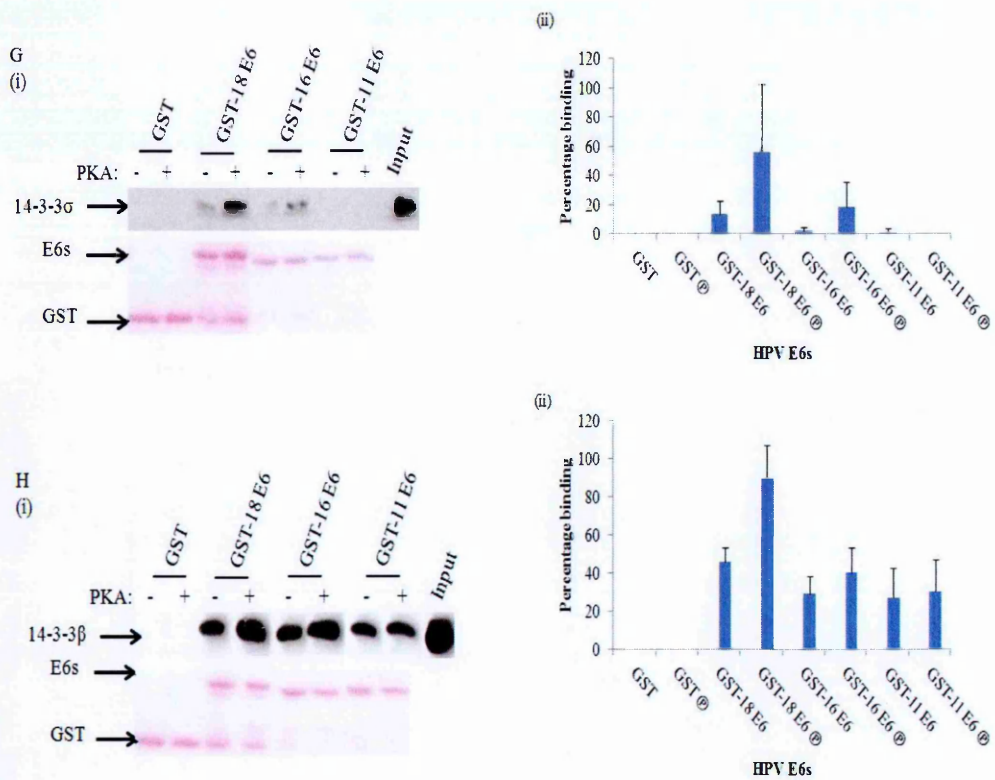
### **3.7 HPV E6s interact with multiple 14-3-3 proteins in a phosphorylation-dependent manner.**

As mentioned earlier, there are 7 mammalian 14-3-3 isoforms which often exist as homo- or heterodimers (Jones *et al*, 1995a). The  $\zeta$  isoforms interact strongly with HPV E6s and whether the other 14-3-3 isoforms can also interact with E6 in a similar manner is unknown. In order to understand this, similar direct binding assays were carried out with all of the other purified His-tagged 14-3-3 isoforms. The results showed that phosphorylation of HPV-18 and -16 E6 by PKA confers an increased interaction with 14-3-3 $\gamma$  [Figure 20C (i) and (ii)],  $\epsilon$  [Figure 20D(i) and (ii)],  $\eta$  [Figure 20E(i) and (ii)] and  $\tau$  [Figure 20F(i) and (ii)] isoforms, and a marginal increase was observed for the  $\beta$  isoform [Figure 20H(i) and (ii)]. A strong interaction between 14-3-3 $\sigma$  and HPV-18E6 was observed, as shown in Figure 20G(i) and (ii), however, the interaction between  $\sigma$  and HPV-16E6 is somewhat







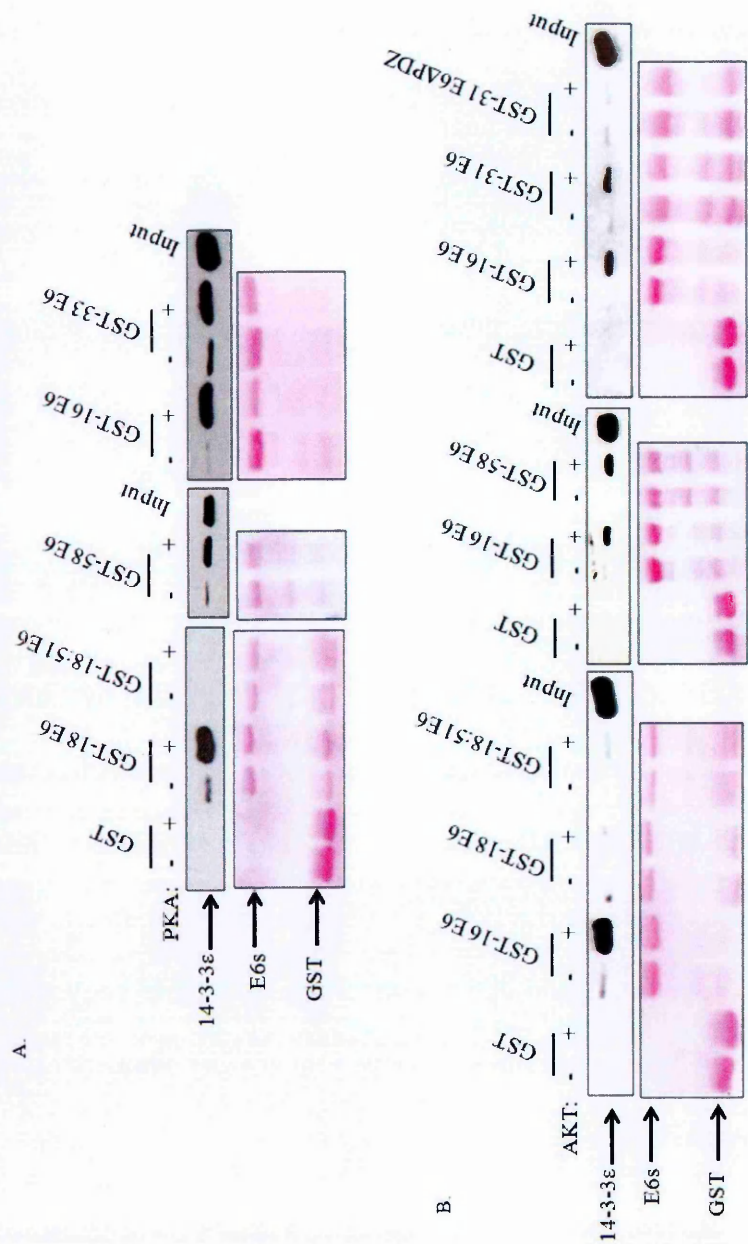


**Figure 20. Interaction between HPV E6 and 14-3-3 proteins is direct.** The indicated purified GST fusion proteins were either untreated (-) or subjected to phosphorylation with PKA (+) in the presence of cold ATP. These were then incubated with purified His-tagged 14-3-3 isoforms: ζ [A (i)], γ [C (i)], ε [D (i)], η [E(i)], τ [F(i)], σ [G(i)] and β [H(i)]. **B.** The indicated purified GST fusion proteins were also either untreated (-) or incubated with AKT (+) in the presence of cold ATP and were then incubated with purified His-tagged 14-3-3ζ. After extensive washing the bound protein was detected by western blotting using anti-His antibody (upper panel). The lower panels show the Ponceau stain of the nitrocellulose membranes. The quantitations from at least three independent assays for the individual isoforms are shown in the respective Panel (ii).

weaker. Interestingly, there is no interaction between most of the 14-3-3 isoforms and LR HPV-11 E6, except for 14-3-3 $\beta$  [Figure 20H(i) and (ii)].

Since it was shown that different HPV E6 oncoproteins were subject to different degrees of phosphorylation, I was also interested in investigating the potential pattern of associations between these E6 oncoproteins and 14-3-3. In order to do this the different GST-E6 fusion proteins were purified and phosphorylated using either AKT or PKA. The E6 proteins were then incubated with commercially available, affinity purified His-tagged 14-3-3 $\epsilon$  and, following extensive washing, bound 14-3-3 was detected by western blotting. The results in Figure 21A demonstrate that HPV-18, -16 and -58 E6 proteins, which are good substrates of PKA, are also able to interact strongly with 14-3-3 $\epsilon$  when phosphorylated by PKA. Likewise, HPV-16, -58 and -31 E6 proteins which are good substrates of AKT, also interact strongly with 14-3-3 $\epsilon$  (Figure 21B) when phosphorylated by AKT. Meanwhile, HPV-18:51 E6, which is a poor substrate for both PKA and AKT, did not bind to 14-3-3 $\epsilon$  (Figure 21A and B). Taken together these results demonstrate that the E6 PBMs of multiple HPV types are dual functional, and, depending upon the phosphorylation status, can confer interaction with either PDZ domain-containing proteins or members of the 14-3-3 family of proteins.

Next, I was interested in investigating the ability of HPV-18 E6 to interact with different 14-3-3 isoforms *in vivo*. To assess this, HEK293 cells were transfected with HA-tagged HPV-18 E6 and samples were extracted after 24 hrs and immunoprecipitated with anti-HA conjugated agarose beads. Co-

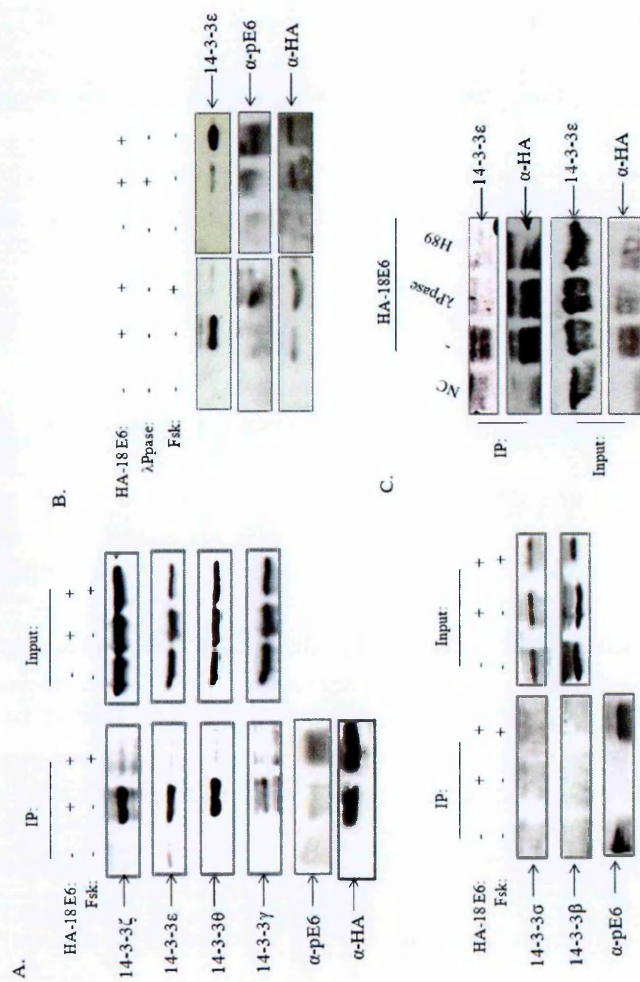


**Figure 21. Diverse HPV E6 oncoproteins interact with 14-3-3 in a phosphorylation dependent manner.** Different HPV E6 GST fusion proteins were purified and phosphorylated with either PKA (Panel A) or AKT (Panel B) in the presence of cold ATP. They were then incubated with purified 14-3-3ε and, after extensive washing, the bound His-tagged 14-3-3ε was detected by western blotting with anti-6X His antibody. The upper panels show the results of the western blots, whilst the lower panels show the Ponceau stain of the nitrocellulose membranes confirming equal levels of protein loading. Arrows show the location of the relevant proteins.

immunoprecipitated 14-3-3 was then detected by western blotting. The results, in Figure 22A, show significant levels of interaction between HPV-18 E6 and 14-3-3 $\zeta$ ,  $\epsilon$  and  $\theta$ , and a much weaker association with 14-3-3 $\gamma$ , while no interaction was detected with  $\sigma$  and  $\beta$  isoforms. Fsk was included in the assay to determine whether this would increase the degree of association between E6 and the different 14-3-3 isoforms. Surprisingly, Fsk treatment appears to strongly inhibit the interaction between 14-3-3 and E6 (Figure 22A and B). The most likely explanation is that the very high levels of active PKA also phosphorylate 14-3-3, which, in turn, is known to inhibit the ability of 14-3-3 to recognize its substrates (Gu *et al*, 2006), and therefore results in a loss of interaction with E6.

To investigate further whether the interaction between E6 and 14-3-3 is phosphorylation-dependent, the cell extracts were incubated with  $\lambda$  phosphatase or cells were exposed to treatment with H89 which is a PKA inhibitor, prior to performing the co-immunoprecipitation. The results in Figure 22B and C again demonstrate a clear co-immunoprecipitation of 14-3-3 $\epsilon$  with HPV-18 E6, but this interaction is abolished following treatment of the cells with H89, and treatment of the cell extract with  $\lambda$  phosphatase. Taken together these results demonstrate that HPV-18 E6 can interact with multiple 14-3-3 isoforms, and that this interaction is phosphorylation-dependent.





**Figure 22. HPV-18 E6 interacts with multiple 14-3-3 isoforms *in vivo* in a phosphorylation dependent manner.** A. HEK293 cells were transfected with HA-tagged HPV-18 E6 expression plasmid. After 24hrs the cells were extracted and immunoprecipitated with anti-HA-conjugated agarose beads, and co-precipitating 14-3-3 isoforms were detected by western blotting. Also shown is the anti-HA (α-HA) blot for total E6 and anti-phospho-E6 (pE6) and the right hand panels show the protein inputs for the different 14-3-3 isoforms. **B and C.** HEK293 cells were either mock transfected (NC) or transfected with HA-tagged HPV-18 E6 and after 24hrs cell extracts were either untreated (-) or treated (+) with λ phosphatase (λPase) for 15 minutes or with forskolin (Fsk) (Panel B) or H89 (Panel C) as indicated and then immunoprecipitated with anti-HA conjugated agarose beads. Bound 14-3-3ε was detected by western blotting. Also shown is the anti-HA blot for total E6 and anti-phospho-E6 (pE6).

### 3.8 HPV-18 E6 does not degrade 14-3-3

HPV-18 E6 targets many cellular proteins for degradation and it was of obvious interest to investigate whether E6 could affect the levels of 14-3-3 protein. In order to assess this in the cells, HEK 293 cells were transiently transfected with Flag-tagged 14-3-3 $\zeta$  and HA-tagged HPV-18 E6, with HA-tagged hDlg included as control. After 24 hrs, the cells were either left untreated or treated with H89 or Fsk. As can be seen in Figure 23, HPV-18 E6 degrades hDlg most efficiently when the cells were treated with H89, where the majority of E6 is not phosphorylated. However, Fsk treatment which increases phosphorylation of E6 inhibits hDlg degradation. This is in agreement with the previous reports showing that HPV-18 E6 association with hDlg is dependent upon the E6 phospho-status of E6 (Kühne *et al*, 2000). In contrast, there is no change in the levels of 14-3-3 $\zeta$  regardless of the presence of HPV-18 E6 or the H89 and Fsk treatments. Taken together, these results indicate that 14-3-3 $\zeta$  is not a degradation target of HPV-18 E6.

### 3.9 HPV-18 E6 is phosphorylated in a cell cycle-dependent manner.

The above results indicate that HPV-18 E6 can be phosphorylated by PKA, but that in asynchronously growing cells the levels of E6 phosphorylation are very low. This suggests that E6 phosphorylation might alter with the phase of the cell cycle. To investigate this, a series of *in vitro* phosphorylation assays on GST-18 E6 was performed, using cell extracts from cells that were harvested at different phases of the cell cycle. To verify that the phosphorylation was occurring within the E6 PBM, the GST-18 E6

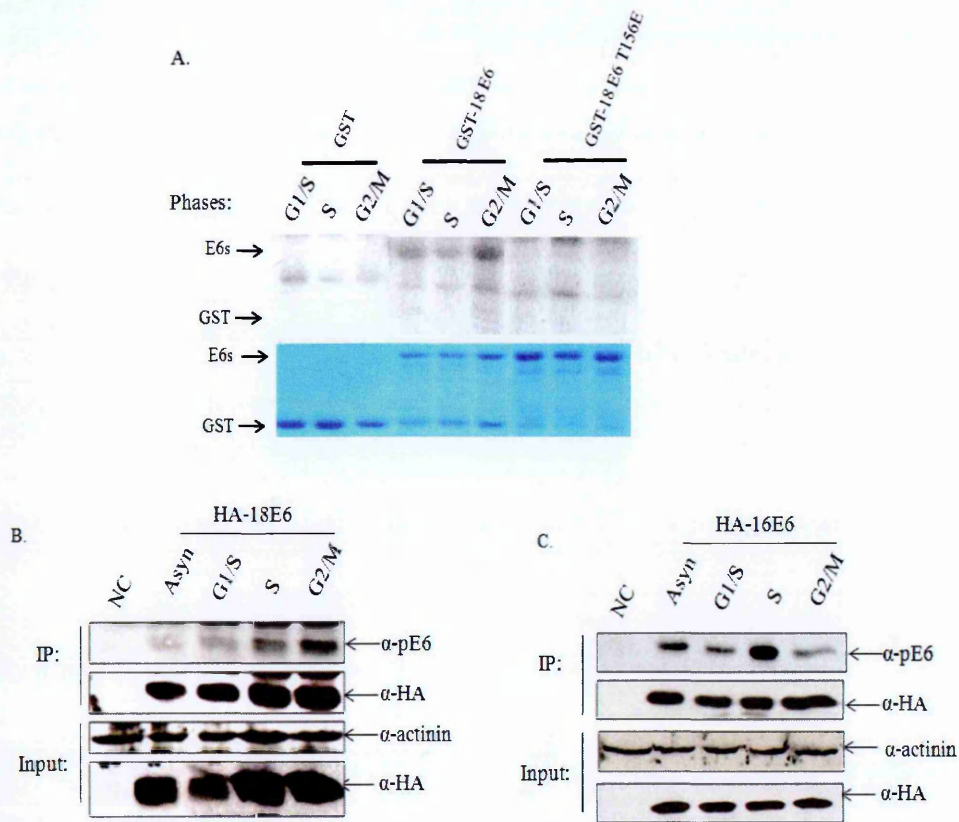


**Figure 23. 14-3-3 is not a degradation target of HPV-18 E6.** HEK293 cells were either mock transfected or transfected with Flag-14-3-3ζ alone, or co-transfected with HA-tagged HPV-18 E6 at a ratio of 3:1 (upper panel). As controls, HEK293 cells were also either mock transfected or transfected with HA-tagged hDlg, or co-transfected with HA-tagged HPV-18 E6 at a ratio of 3:1 (lower panel). As transfection efficiency control, LacZ was also transfected in the cells. After 24 hrs, the cells were either left untreated (N), or treated with H89 (H) or Forskolin (F). These cells were collected and analysed by western blotting using anti-Flag and anti-HA antibodies to detect 14-3-3 and hDlg respectively. Also included was the loading control using β-galactosidase (β-gal) antibody.

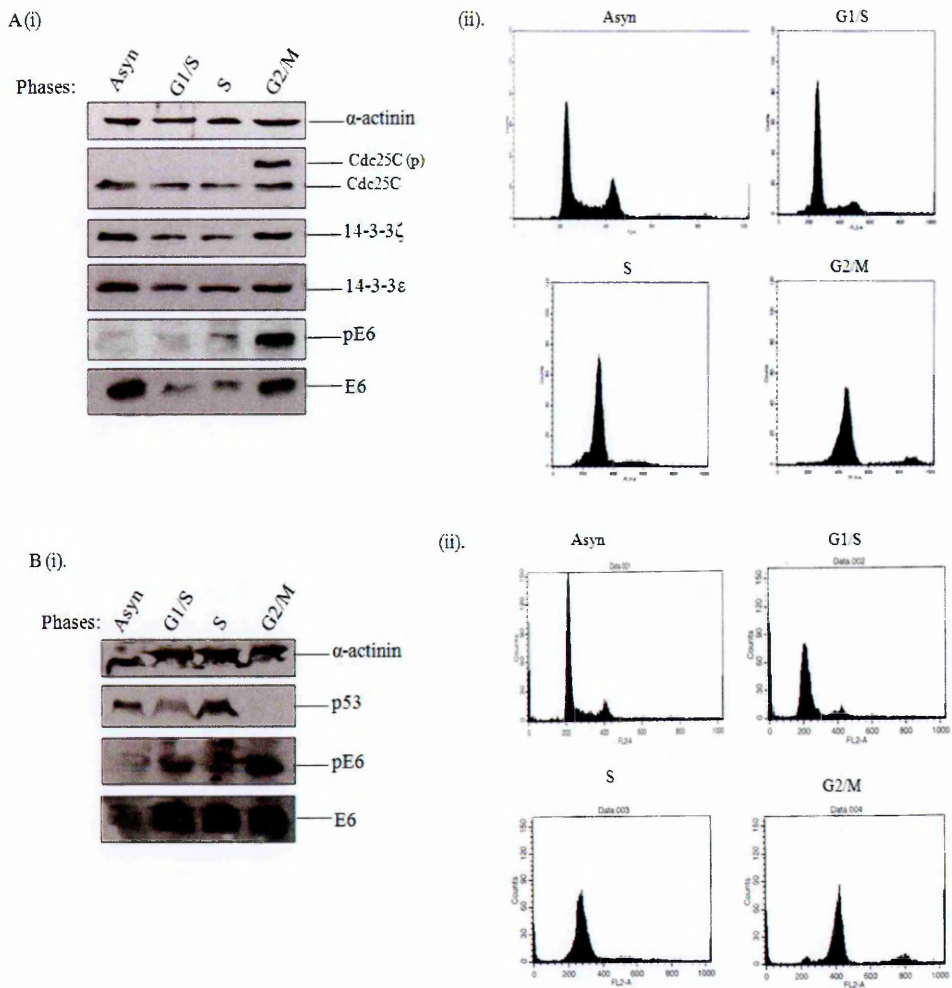


T156E mutant, which cannot be phosphorylated by either PKA or AKT, was also included. Following incubation of the fusion proteins with the cell extracts and radiolabelled ATP, the fusion proteins were separated by SDS-PAGE, and labelled protein was monitored by autoradiography. The results in Figure 24A show that E6 is phosphorylated by cell extracts obtained from cells in G2/M, whereas low levels of phosphorylation are seen using extracts from G1/S or S phase cells.

In order to confirm this result, I then proceeded to investigate the pattern of E6 phosphorylation *in vivo*. HEK293 cells were transiently transfected with HA-tagged HPV-18 and -16 E6 and the cells were synchronized in G1/S, S and G2/M phases of the cell cycle. These cells were then extracted and total E6 immunoprecipitated using anti-HA conjugated agarose beads. Bound phospho-E6 was then detected by western blotting. As can be seen from Figure 24B, the highest levels of HPV-18 E6 phosphorylation occur in the G2/M phase of the cell cycle (Figure 24B), while HPV-16 E6 is most heavily phosphorylated during S phase, although it is phosphorylated throughout the cell cycle (Figure 24C). To monitor the phosphorylation pattern of endogenous E6, HeLa cells were synchronized and total cell extracts then subjected to western blot analysis using either anti-E6 monoclonal antibody to detect total E6 or the  $\alpha$ -pE6 antibody. The cell cycle profile was verified by FACS analysis which is shown in Figure 25A (ii) and B (ii). As can be seen from Figure 25A, Cdc25C is phosphorylated during G2/M phase, which is in agreement with the previous studies (Peng *et al*, 1997). Consistent with the transient expression assay, the majority of



**Figure 24. Cell cycle dependent phosphorylation of HPV-18 E6. A.** Purified HPV-18 E6 and HPV-18 E6 T156E GST fusion proteins and GST alone were incubated with cell lysates from cells synchronised at different phases of the cell cycle ( $G1/S$ ,  $S$  and  $G2/M$ ), together with  $^{32}P$ - $\gamma$ -ATP as indicated. Proteins were subjected to SDS-PAGE and autoradiography. The upper panel shows the autoradiogram and the lower panel shows the Coomassie stain of the gel. Arrows indicate the relevant GST proteins. **B and C.** HEK293 cells were either transiently mock transfected (NC) or transfected with the indicated HA-tagged HPV-18 E6 (Panel B) or HA-tagged HPV-16 E6 (Panel C) expression plasmid. After 24 hrs the cells were synchronised at different cell cycle phases as indicated, or left asynchronous (Asyn). The cells were harvested and subjected to immunoprecipitation using anti-HA conjugated agarose beads. The presence of E6 was then detected by western blotting using either the anti-phospho-E6 specific antibody (upper panels) to detect phosphorylated E6 or the anti-HA antibody to detect total E6.



**Figure 25. Phospho-regulation and turn-over of HPV-18 E6 occurs in a cell cycle dependent manner in HeLa cells. A(i) and B(ii).** HeLa cells were synchronised using Aphidicolin (G1/S) or 5 hrs release from Aphidicolin (S), or 18 hrs of Nocodazole (G2/M), or left asynchronous (Asy) (Panel A). Alternatively, the cells were subjected to double thymidine block, and cells were collected at 0 hrs (G1/S), 5 hrs (S), or 9 hrs (G2/M) post release from double thymidine treatment (Panel B). These cells were harvested and the levels of E6 ascertained by western blotting using anti-phospho-E6 ( $\alpha$ -pE6) specific antibody and total E6 detected using anti-HPV-18 E6 monoclonal antibody. The upper panel shows the  $\alpha$ -actinin loading control. Also included are Cdc25C, 14-3-3 $\zeta$  and  $\epsilon$ . Cdc25C(p) represents phosphorylated form of the Cdc25C during the G2/M phase of the cell cycle. **A(ii) and B(ii).** FACS analysis of asynchronous (Asyn) or synchronised HeLa cells at different cell cycle phases as indicated.

HPV-18 E6 phosphorylation also takes place during the G2/M phase of the cell cycle. The level of total E6 protein is low in the G1/S and S phase of the cell cycle. The levels of 14-3-3 proteins were also studied to understand if their steady state levels can be affected by phospho-E6 across the cell cycle. The results in Figure 25A(i) showed no significant change to the levels of 14-3-3 proteins across the cell cycle. This again indicates that phospho-E6 does not affect the steady state levels of 14-3-3 proteins. To exclude any possibility that these pattern of expression might be due to Aphidicolin and Nocodazole treatments, I repeated the analysis using a double thymidine block. HeLa cells were treated with thymidine for 16 hrs, then released from thymidine for 9 hrs and the cells were then re-treated with thymidine for 16 hrs. Following this, the cells were harvested at 0 hrs (G1/S), 5 hrs (S) and 9 hrs (G2/M). The samples were then analysed by western blotting to detect the levels of phospho-E6, total E6 and p53 proteins. As can be seen from Figure 25B, the highest levels of phospho-E6 were detected in G2/M phase (Figure 25B), although there is little change in the levels of total E6 in the different cell cycle phases.

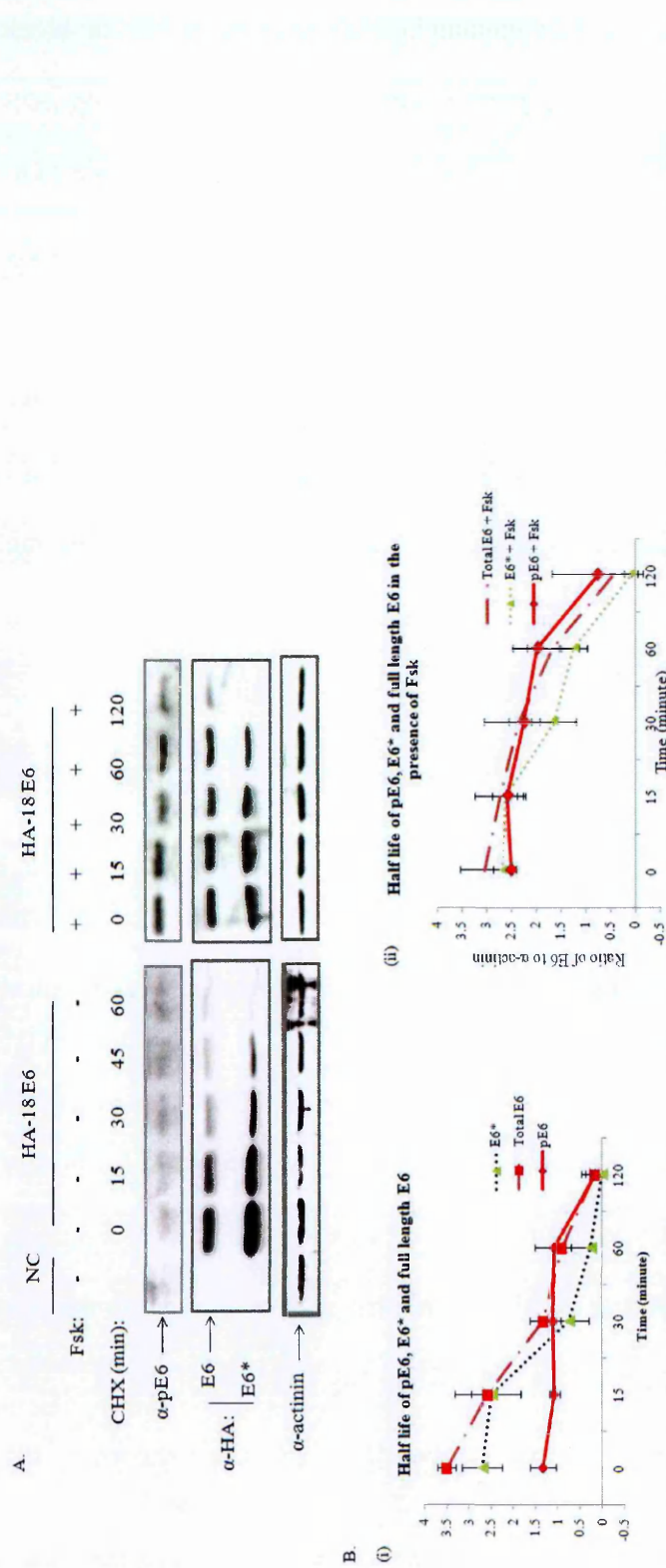
### **3.10 Phosphorylation regulates E6 stability**

Previous studies had shown that the E6 PBM might contribute towards maintaining E6 stability (Nicolaidis *et al*, 2011), although the precise mechanism is still unclear, with both PDZ and 14-3-3 association being implicated. In order to investigate this, cells were transfected with HA-tagged HPV-18 E6 and the cells were either left untreated or treated with

Fsk. After 24 hrs, the cells then incubated with cycloheximide to block protein synthesis, and levels of E6 protein were assessed by harvesting cells at different time-points. HA-18 E6 was immunoprecipitated using anti-HA-conjugated agarose beads and the levels of total and phospho-E6 were ascertained by western blotting. As can be seen from Figure 26A and the quantitation shown in Figure 26B, phospho-E6 levels in the non-Fsk treated cells were low, but the half life is extended in comparison with that of the total E6 and E6\*, which appears to have a half life of around 30 minutes under these conditions. In contrast, when the cells are stimulated with Fsk, the levels of phospho-E6 increased dramatically. In addition, the turnover of this phospho-E6 remains relatively unchanged, with a half-life of around 90 minutes. However, there is a concomitant extension in the half-life of the total E6 and E6\*, similar to that seen with the phospho-E6. This demonstrates that phosphorylation of HPV-18 E6 results in a significant increase in the stability of the E6 oncoprotein.

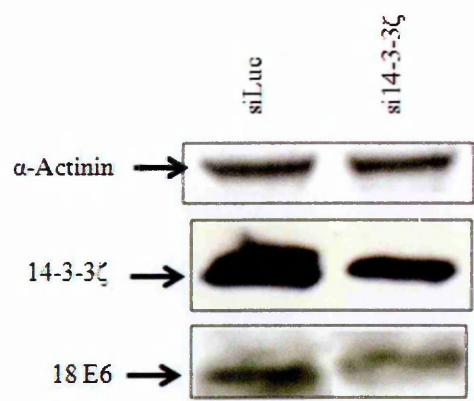
To investigate whether 14-3-3 might play any role in maintaining the steady state levels of E6, siRNA depletion of 14-3-3 $\zeta$  was performed in HeLa cells. After 72 hrs, the total extract of these samples was analysed by western blotting. As can be seen from Figure 27A, downregulation of 14-3-3 $\zeta$  also resulted in a modest decrease in the levels of HPV-18 E6, as shown by the quantification in Figure 27B. From the *in vitro* (Figure 19 and 20) and *in vivo* (Figure 22) binding assays, it is known that the  $\epsilon$  and  $\theta$  isoforms bind strongly to HPV-18 E6. Downregulation of multiple 14-3-3 isoforms might have a more detrimental effect on the levels of HPV-18 E6. Taken together,



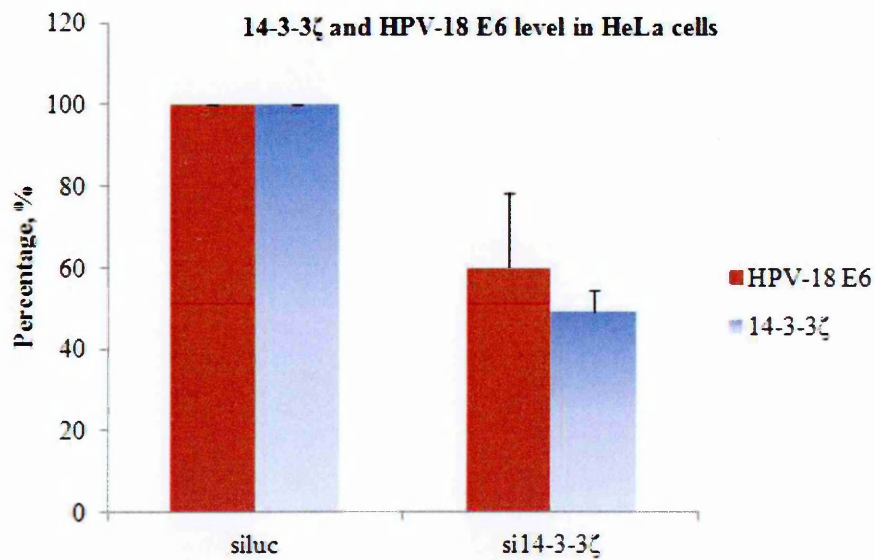


**Figure 26. Phosphorylation extends the half-life of HPV-18 E6.** **A.** HEK293 cells were mock transfected (NC) or transiently transfected with HA-tagged HPV-18 E6 expression plasmids as indicated, in the presence (+) or absence (-) of forskolin (Fsk). After 24hrs the cells were incubated with cycloheximide (CHX) for different times as indicated. The cells were harvested and subjected to immunoprecipitation using anti-HA conjugated agarose beads. The presence of E6 was then detected by western blotting using either the anti-phospho-E6 specific ( $\alpha$ -pE6) antibody (upper panels) to detect phosphorylated E6 or the anti-HA antibody to detect total level of E6 and E6\*. Also shown is the input control for the immunoprecipitation of  $\alpha$ -actinin. **B.** Quantitation from at least three independent experiments showing the levels of phospho-E6 and total E6 in relation to the  $\alpha$ -actinin control in the absence (i) or presence (ii) of Fsk. Error bars represent standard deviations.

A.



B.



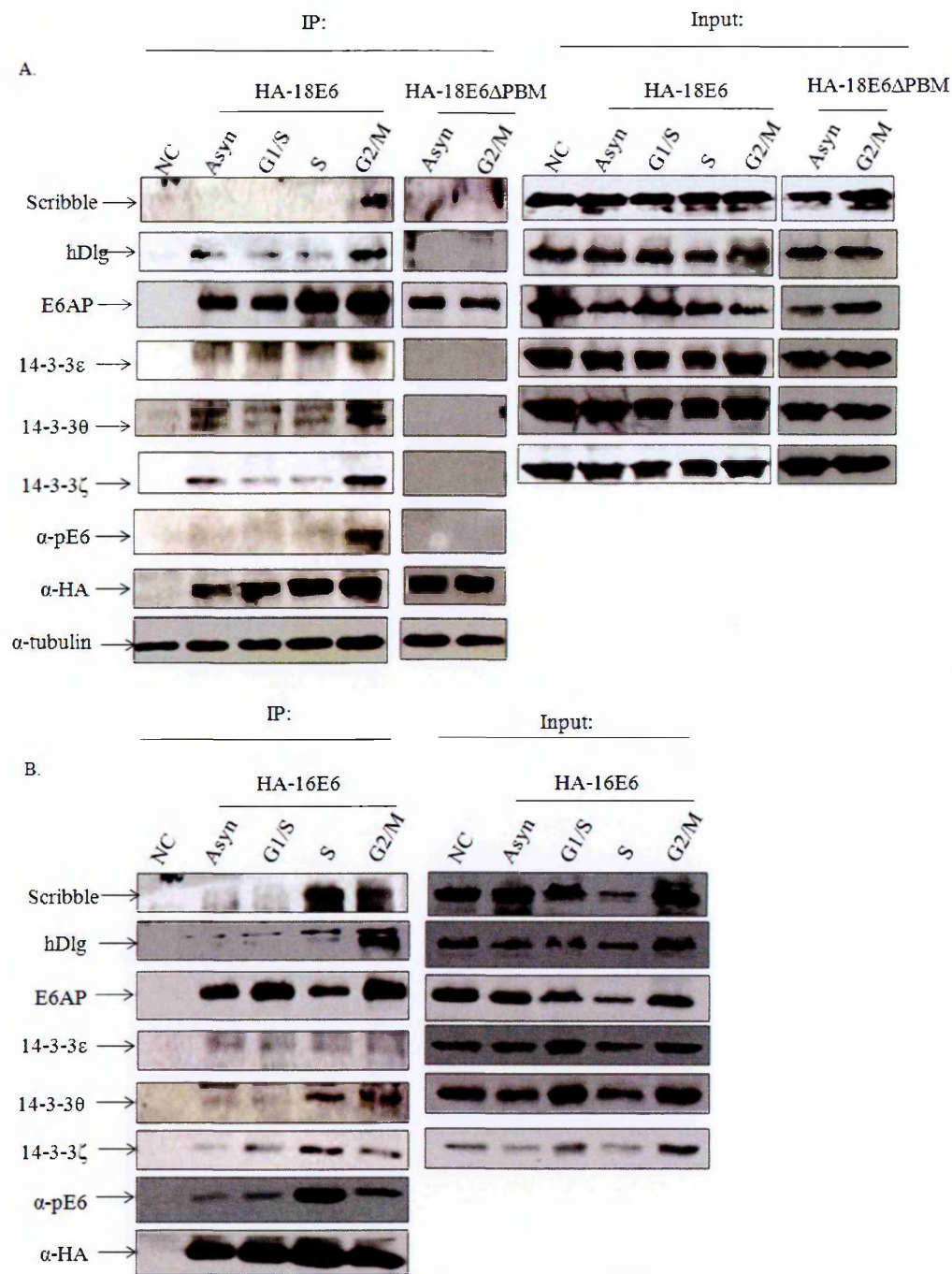
**Figure 27. 14-3-3 $\zeta$  contributes towards maintaining HPV-18 E6 steady state levels.** A. HeLa cells were transfected with control siRNA (siLuc) and siRNA against 14-3-3 $\zeta$  (si14-3-3 $\zeta$ ). After 72hrs the cells were harvested and the level of E6 was ascertained by western blotting using anti-HPV-18 E6 monoclonal antibody. Also shown are the levels of 14-3-3 $\zeta$  and the loading control  $\alpha$ -actinin. B. The quantitation from at least three independent experiments showing the 14-3-3 $\zeta$  knockdown and the effects upon E6 levels.

these studies indicate that both phosphorylation and 14-3-3 interactions can be expected to play a role in the regulation of E6 stability.

### **3.11 HPV E6 recognition of 14-3-3 family members and PDZ substrates occurs during distinct phases of the cell cycle.**

The above results demonstrate that phosphorylation of E6 can affect E6 turnover and its capacity to interact both with 14-3-3 family members and PDZ domain-containing substrates. Since phosphorylation of E6 varies throughout the cell cycle, I next wanted to determine if this affects E6 substrate recognition. To do this, HEK293 cells were transfected with HA-tagged HPV-16 E6, HPV-18 E6 and HPV-18 E6  $\Delta$ PBM expression plasmids and the cells were synchronized in G1/S, S and G2/M. Three hours prior to harvesting, cells were treated with CBZ and HA-HPV 18 E6 was immunoprecipitated with anti-HA conjugated agarose beads. Bound complexes were then analysed by western blotting and the results are shown in Figure 28A (HPV-18 E6) and Figure 28B (HPV-16 E6). As a control, co-immunoprecipitation of E6AP was monitored, and as can be seen this appears to be in complex with HPV-16 E6 and HPV-18 E6 throughout the cell cycle and, as expected, the HPV-18 E6  $\Delta$ PBM mutant also retains ability to interact with E6AP. The levels of E6 increase slightly as the cells progress into S and G2/M, and there was a clear increase in the levels of HPV-18 E6 phosphorylation during G2/M, and, as expected, this increase in phosphorylation was not detected with the E6  $\Delta$ PBM mutant. Association between HPV-18 E6 and 14-3-3 $\epsilon$ ,  $\zeta$  and  $\theta$  isoforms also shows a clear cell





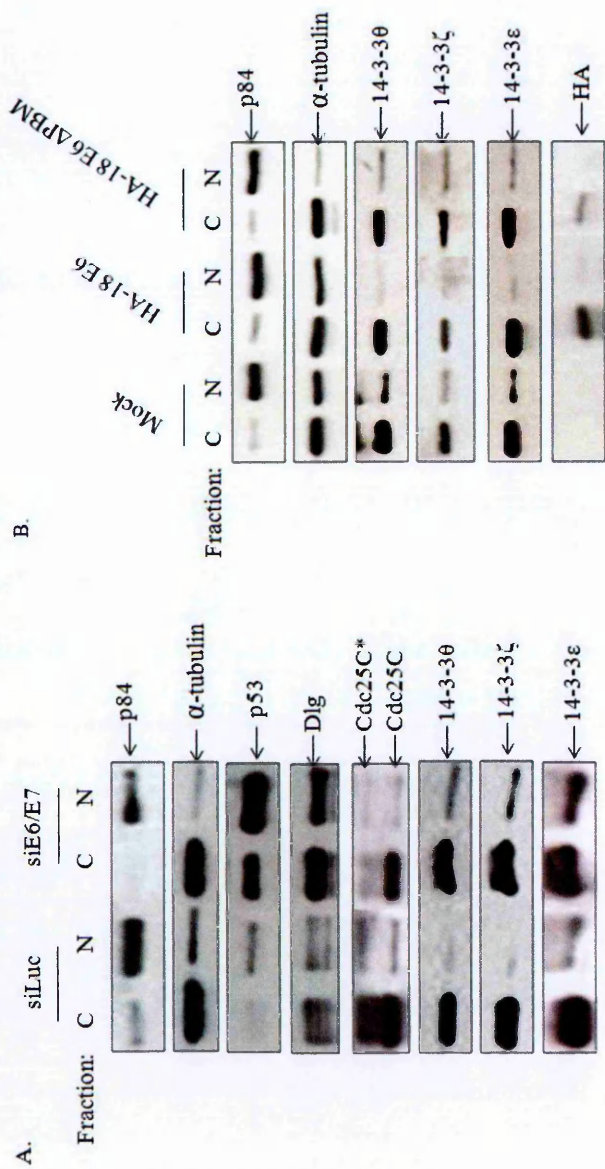
**Figure 28. HPV-18 and HPV-16 E6 interact with 14-3-3 in a cell cycle and PBM-dependent manner.** HEK293 cells were mock transfected (NC) or transiently transfected with HA-tagged HPV-18 E6, HA-tagged HPV-18 E6ΔPBM (Panel A) or HA-tagged HPV-16 E6 (Panel B) and cells were either grown asynchronously (Asyn) or harvested during G1/S, S or G2/M as indicated. Cells were then extracted and immunoprecipitated using anti-HA conjugated agarose beads and co-immunoprecipitating proteins were analysed by western blotting. The right hand panels show the protein inputs used in each of the assays. Note the increase in 14-3-3 associated with HPV-18 E6 in G2/M and the increase in 14-3-3 associated with HPV-16 E6 in S phase.

cycle regulation, with a strong increase in the interactions in G2/M phase cells. Interestingly, hDlg and Scribble also showed a very similar pattern of co-immunoprecipitation, with a significant increase in the amount of hDlg and Scribble complexed with HPV-18 E6 as the cells enter G2/M. Not surprisingly, all associations of E6 with hDlg and 14-3-3 proteins are lost upon mutation of the E6 PBM, confirming the specificity of these interactions.

In the case of HPV-16 E6 (Figure 28B), the interactions are somewhat different and reflect differences in the patterns of HPV-16 E6 and HPV-18 E6 phosphorylation. Indeed, in the cell cycle analysis, the bulk of HPV-16 E6 phosphorylation occurs during S phase, although there was still a significant amount of phosphorylated HPV-16 E6 detected in G2/M. In concordance with this it is also clear that the majority of 14-3-3 interactions with HPV-16 E6 also occur during S phase, when HPV-16 E6 is most heavily phosphorylated. In this case HPV-16 E6 appears to interact preferentially with the 14-3-3 $\zeta$  and  $\theta$  isoforms, but not with the  $\epsilon$  isoform. Furthermore, 14-3-3 interactions are also still apparent during G2/M, which is consistent with there still being readily detectable levels of E6 phosphorylation. Intriguingly, a very strong association of HPV-16 E6 with hDlg was detected during G2/M phase, whilst a strong association of HPV-16 E6 with Scribble was also detected during S phase and this was somewhat weaker during G2/M phase of the cell cycle. All these results indicate the differential phospho-regulation of HPV E6 and their preference in protein targeting during different phases of cell cycle.

### 3.12 HPV-18 E6 alters 14-3-3 subcellular distribution.

A major function of 14-3-3 is to regulate the subcellular distribution of its numerous binding partners (Muslin & Xing, 2000; Aitken, 2006). Therefore, association of E6 with 14-3-3 may potentially have an effect on the subcellular distribution of 14-3-3. Since the association between E6 and 14-3-3 occurs primarily during G2/M, I focused on any changes during this phase of the cell cycle. HeLa cells were transfected with siRNA luciferase as a control or siRNA E6/E7. After 48 hrs Nocodazole was added to induce a G2/M population of cells, where E6 is expected to be most heavily phosphorylated. After a further 18 hrs the cells were harvested and extracts separated into nuclear and cytoplasmic fractions. The patterns of 14-3-3, hDlg and p53 expression was then ascertained by western blotting and the results obtained are shown in Figure 29A. As can be seen, there is a dramatic rescue in the levels of p53 in the nuclear fraction following ablation of HPV-18 E6/E7 expression. Likewise, hDlg levels were particularly low during G2/M, consistent with the results in Figure 28, and there is a corresponding large increase in hDlg levels in both the cytoplasmic and nuclear fractions upon ablation of HPV E6/E7 expression. In the case of 14-3-3, all three 14-3-3 isoforms analysed show a predominantly cytoplasmic distribution, with very low levels of 14-3-3 detected within the nucleus. However, following ablation of E6/E7 expression, the levels of these 14-3-3 isoforms within the nuclear fraction were increased (Figure 29A). In order to determine whether these effects were related to the ability of E6 to interact with 14-3-3, HaCaT cells were transfected with wild type HPV-18 E6 and the HPV-18E6  $\Delta$ PBM mutant.



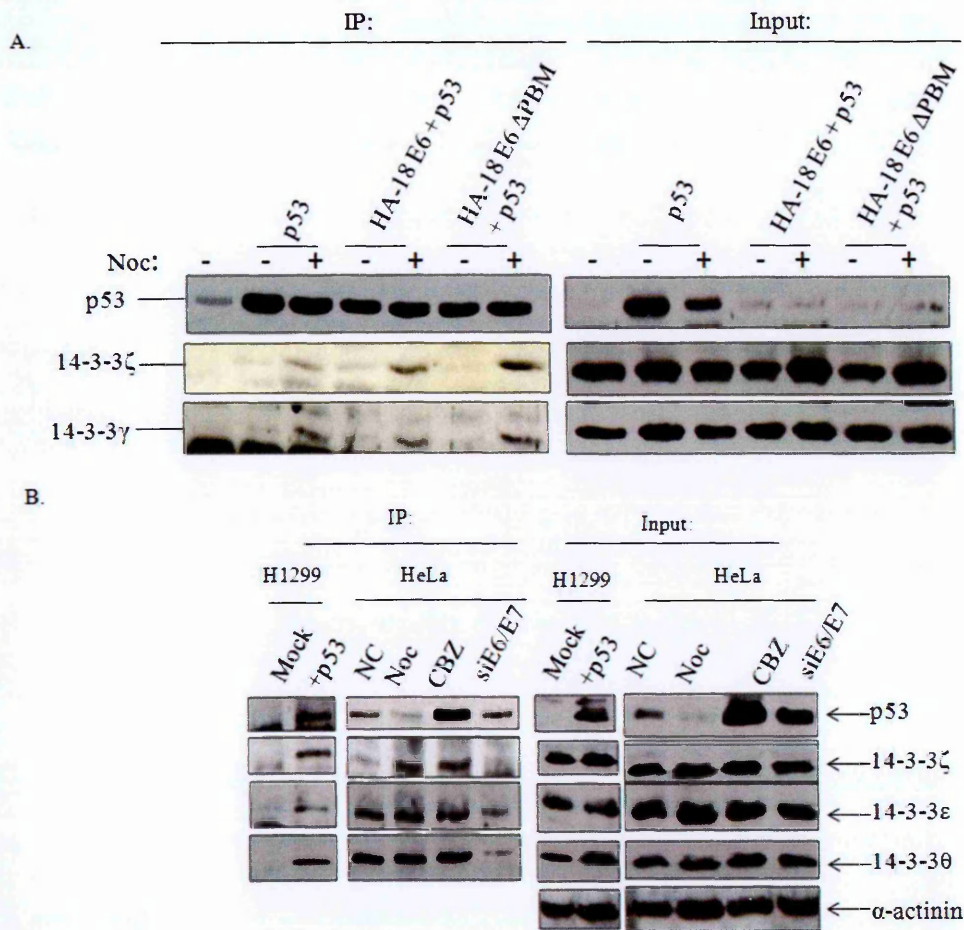
**Figure 29. HPV-18 E6 alters nuclear accumulation of 14-3-3 following Nocodazole treatment.** A. HeLa cells were transfected with siRNA against Luciferase (siLuc) or against HPV-18 E6/E7 (siE6/E7) and after 48 hrs Nocodazole was added for a further 18 hrs. The cells were then harvested and divided into cytoplasmic (C) or nuclear (N) fractions. The pattern of expression of the different 14-3-3 isoforms were then assessed by western blotting. Controls include p84 and  $\alpha$ -tubulin serving as loading controls for the nuclear and cytoplasmic fractions respectively. B. HaCaT cells were either mock transfected (Mock) or transfected with HA-tagged wild type HPV-18 E6 or the  $\Delta$ PBM mutant as indicated. After 24 hrs Nocodazole was added for a further 18 hrs and the cells were then harvested and divided into cytoplasmic (C) or nuclear (N) fractions. The pattern of expression of the different 14-3-3 isoforms and HA-tagged E6 were then assessed by western blotting. p84 and  $\alpha$ -tubulin served as loading controls for the nuclear and cytoplasmic fractions respectively.

After 24hrs, Nocodazole was added and after a further 18 hrs the cells were harvested and separated into cytoplasmic and nuclear fractions. The results obtained are shown in Figure 29B and demonstrate that overexpressed wild type HPV-18 E6 can reduce the levels of nuclear forms of 14-3-3 $\epsilon$ ,  $\theta$  and  $\zeta$  that are seen following exposure to Nocodazole and this is in part dependent upon an intact PBM.

### 3.13 HPV-18 E6 inactivates p53 function depending upon 14-3-3.

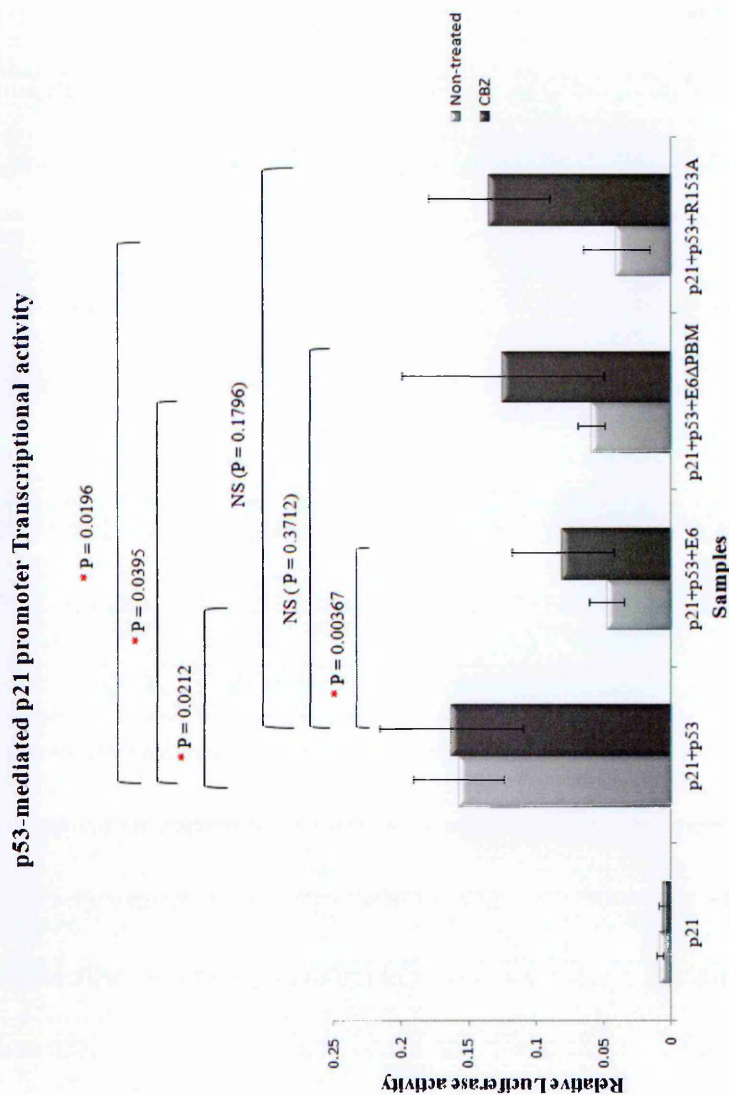
Although p53 is degraded by HPV E6, there are nonetheless readily detectable levels of p53 in HeLa cells, which is inactive. The mechanism of this p53 inactivation is unclear but 14-3-3 has a role in regulating p53 DNA binding capability and transactivation activity in the nucleus (Waterman *et al*, 1998; Rajagopalan *et al*, 2010). Furthermore, recent studies have indicated that loss of E6-PBM function can be compensated in part by the loss of p53, suggesting that some of E6's PBM related activities might involve perturbation of p53 function (Brimer & Vande Pol, 2014). I therefore reasoned that E6 might perturb the 14-3-3/p53 interaction in a PBM-dependent manner. In order to investigate this, I first performed p53-14-3-3 co-immunoprecipitation assays from extracts of p53-null H1299 cells following transfection of Flag-tagged p53 with either wild-type HPV-18 E6 or with the E6  $\Delta$ PBM mutant in the presence and absence of Nocodazole. The results obtained in Figure 30A indicate a strong increase in complex formation between 14-3-3 and p53 upon Nocodazole treatment, even in the presence of E6. A similar analysis was also performed in HeLa cells, and as





**Figure 30. p53 interacts with 14-3-3 during the G2/M phase of cell cycle.**  
**A.** H1299 cells were either transfected with Flag-tagged p53 alone, or co-transfected with HA-tagged HPV-18 E6 or  $\Delta$ PBM mutant. After 24 hrs, cells were either untreated (-) or treated (+) with Nocodazole for further 18 hrs and 3 hrs prior to harvesting cells were also treated with proteasome inhibitor CBZ. Cells were then extracted and immunoprecipitated using anti-Flag conjugated agarose beads and co-immunoprecipitating proteins were analysed by western blotting. The right hand panels show the protein inputs used in each of the assays. **B.** HeLa cells were either non-treated (NC) or treated with Nocodazole for 18 hrs, CBZ for 3 hrs or transfected with siRNA against HPV-18 E6/E7 (siE6/E7) as indicated. As control, H1299 cells were either mock transfected (Mock) or transfected with Flag-tagged p53. Cells were extracted and p53 was immunoprecipitated using DO1 anti-p53 monoclonal antibody and protein A sepharose beads. The bound proteins were ascertained by western blotting. The right hand panels show the protein inputs used in each of the assays.

can be seen from Figure 30B, there is again a significant degree of association between p53 and 14-3-3. Indeed, removal of E6/E7 expression does not appear to increase the p53-14-3-3 interaction. These results suggest that in the presence of E6, p53 interaction with 14-3-3 can still occur. The next question was whether p53 was functionally active in these complexes, and in particular whether the E6 PBM function had a role in this aspect. To investigate this, I analysed the p53 activation of the p21 promoter in the presence and absence of HPV-18 E6 wild-type,  $\Delta$ PBM and PKA mutants. Assays were also performed in the absence or presence of CBZ to prevent the E6-induced degradation of p53. The results obtained are shown in Figure 31 and demonstrate a robust activation of the p21 promoter by p53, which in turn is abolished by both wild type and mutant E6 proteins to a significant levels, consistent with their ability to target p53 for degradation. However, in the presence of CBZ, wild-type E6 can still efficiently inhibit p53 transcriptional activity, but there is no significant change to the levels of p21 promoter activity in the presence of both E6 mutants. In addition, in the presence of CBZ, both of the E6 mutants had a significant effect in increasing the p21 promoter activity. These results demonstrate that phosphorylation of the HPV-18 E6 PBM, and hence association with 14-3-3, most likely contribute to the ability of E6 to inhibit p53 transcriptional activity, independent of its ability to target p53 for degradation.



**Figure 31. HPV-18 E6 PBM perturbs p53 transcriptional transactivation activity mediated by 14-3-3.** H1299 cells were transiently transfected with pCMV-Renilla and p21 promoter luciferase expression constructs, or co-transfected with Flag-p53 in the absence or presence of HA-tagged HPV-18 E6, HPV-18 E6ΔPBM or R153A mutants. After 24 hrs, the cells were extracted and luciferase assays were performed to assess the p21 promoter activity mediated by p53. The bar graph represents the average data from at least three independent experiments with standard deviation bars shown. Also shown is the p value of the changes of the relative luciferase activity as indicated; \* indicates statistical significant difference and NS indicates no statistically significant difference.



## CHAPTER 4: DISCUSSION

### 4.1 Differential phosphorylation of HR HPV type E6s.

The presence of a PBM on the carboxy terminus of cancer-causing E6 oncoproteins is an essential element in the ability of these proteins to support the viral life cycle (Lee & Laimins, 2004; Nicolaides *et al*, 2011; Delury *et al*, 2013), to contribute towards cell transformation (Kiyono *et al*, 1997) and to the induction of malignancy (Simonson *et al*, 2005; Nguyen *et al*, 2003). This is partly owing to the ability of the PBM to recognise PDZ-containing proteins, such as hDlg, Scribble and MAGI-1, allowing E6 to target them for degradation in a proteasome-dependent manner. As a result of this, the normal functions and distribution of these proteins are disturbed, and one potential consequence is perturbation of cell polarity. It was discovered some time ago that E6 PBM peptides from the HR HPV types can be phosphorylated by PKA with similar kinetics (Kühne *et al*, 2000). However, in my analyses on full length E6 proteins, I found that phosphorylation by PKA is dissimilar between the E6 oncoproteins of different HPV types. HPV-18 and -58 E6 are the best substrates, followed by HPV-16 E6, HPV-31 E6 is a weaker substrate. These differences might reflect differences in how the HR HPV E6 proteins are regulated by kinases. The other potential candidate kinases predicted for the HPV-18 E6 could be PAK, CamK II and AKT. From my analysis, I found that HPV-18 E6 was a very weak substrate for PAK and CamK II, and this probably did not involve the PBM. HPV-18 E6 was also weakly phosphorylated by AKT, but

this did involve the PBM. The analysis was then extended to study other HPV E6 oncoproteins. AKT can phosphorylate HPV-16 and -58 E6 strongly, and HPV-31 E6 to a lesser extent. The results also showed that HPV-33 and -51 E6 are very poor substrates for both PKA and AKT even though these proteins contain PBM. Interestingly, HPV-51 E6 contains an amino acid "N" instead of "R", located within the kinase recognition motif, whilst HPV-33 E6 contains amino acid "A" instead of "Q" within its PBM (as shown in Figure 10). These may have an effect on their susceptibility to PKA or AKT phosphorylation, whilst for HPV-58 E6, the amino acid "Q" instead of "E" within its PBM does not affect the phosphorylation. However, it is worth noting that HPV-51 E6 used in this study was actually HPV-18 E6, with its C-terminal PBM mutated to mimic the PBM of HPV-51 E6. Therefore, the susceptibility of the full length HPV-51 E6 to PKA and AKT phosphorylation needs to be further verified. It is also worth mentioning that the differential phosphorylation observed among these different types of bacterially purified HPV E6 GST fusion proteins are not due to artifacts, for example, arising from poor protein folding or solubility, as these proteins are able to interact with PDZ domain-containing proteins.

The differential recognition of these different E6s by the different kinases can probably be explained by the fact that kinase-substrate recognition depends on several factors, including the structure of the catalytic cleft that accommodates its substrate, the 4 amino acids on either the N-terminus or C-terminus of the phospho-acceptor site and the docking motifs on the substrate that can be recognised by the interacting domain on the kinase (Ubersax & Ferrell, 2007). In addition, the different susceptibilities of HPV

E6 proteins to different kinases that I have shown in this study, as opposed to the previous study using short peptides (Kühne *et al*, 2000) is most likely explained by the differences in how the kinase catalytic domain can accommodate the phospho-acceptor site of the unstructured 10 amino acid E6 peptides in comparison with the recognition of the full length E6 proteins, which are approximately 40 times bigger than the peptide.

Having defined that HPV-18 E6 is an excellent substrate for PKA phosphorylation, analyses were done to verify that the principal phospho-acceptor site was T156 within the PBM. By introducing a number of point substitutions in HPV-18 E6, including T156D and T156E, mutation of the kinase consensus motif R153A, as well as S82A, which has been predicted to be another potential phospho-acceptor site, it became clear that T156 is indeed the major phospho-acceptor site for HPV-18 E6. To extend these studies to other HPV E6s, the anti-phospho-E6 specific ( $\alpha$ -pE6) antibody was used. As can be seen from the amino acid sequence alignment, this antibody could potentially cross-react with a number of E6 oncoproteins that are phosphorylated within their PBM. It was found that both HPV-16 and -58 E6 are also phosphorylated by PKA and AKT at their PBM, at T156 and T147, respectively, which are equivalent to T156 of HPV-18 E6. In the case of HPV-31 E6, it has two potential phospho-acceptor sites within the last 6 amino acids of its carboxy terminus, this protein is a substrate for both AKT and PKA, but AKT would appear to phosphorylate the downstream T147 within the PBM, whilst PKA phosphorylates HPV-31 E6 at position S82, well away from the PBM. This suggests that HPV-31 E6 may be regulated

very differently from the other HPV E6 oncoproteins analysed, including the PDZ recognition.

From the results obtained, it is quite clear that there are significant variations in the manner in which the different E6 oncoproteins are regulated by different kinases. This raises important questions, firstly about the relevance of these modifications for the normal viral life cycle. In asynchronously growing monolayer culture, HPV-16 E6 is phosphorylated to higher levels than HPV-18 E6. This could be the result of higher levels of AKT in the cells, or it might indicate that HPV-16 E6 can be phosphorylated by other kinases. There are 4 potential phospho-serine/threonine kinase phosphorylation sites (T140, S145, S149 and S150) located in near vicinity of the C-terminal PBM of HPV-16 E6 (Refer to Appendix), and these sites could serve as "priming sites", in which phosphorylation at these sites might favour the accessibility of AKT to T156. However, this possibility remains to be verified. In the context of a differentiating epithelium, AKT levels are higher in the basal cells and suprabasal layer (Menges *et al*, 2006; Segrelles *et al*, 2007). This would suggest that HPV-16 E6 is phosphorylated more extensively throughout its life cycle. Previous studies showed that HPV E1<sup>^</sup>E4 can be phosphorylated by PKA (Bryan *et al*, 2000; Wang *et al*, 2009), and PKA activity appears to be high at the upper intestinal epithelial layers (El-Yazbi *et al*, 2006). This may indicate that HPV-18 E6 phosphorylation may potentially occur in the upper epithelial layers, however, this remains to be verified. Even though both HPV-18 and -16 appear to have similar life cycles, they may behave differently in raft culture models, and this could be a reflection of the higher prevalence of HPV-16

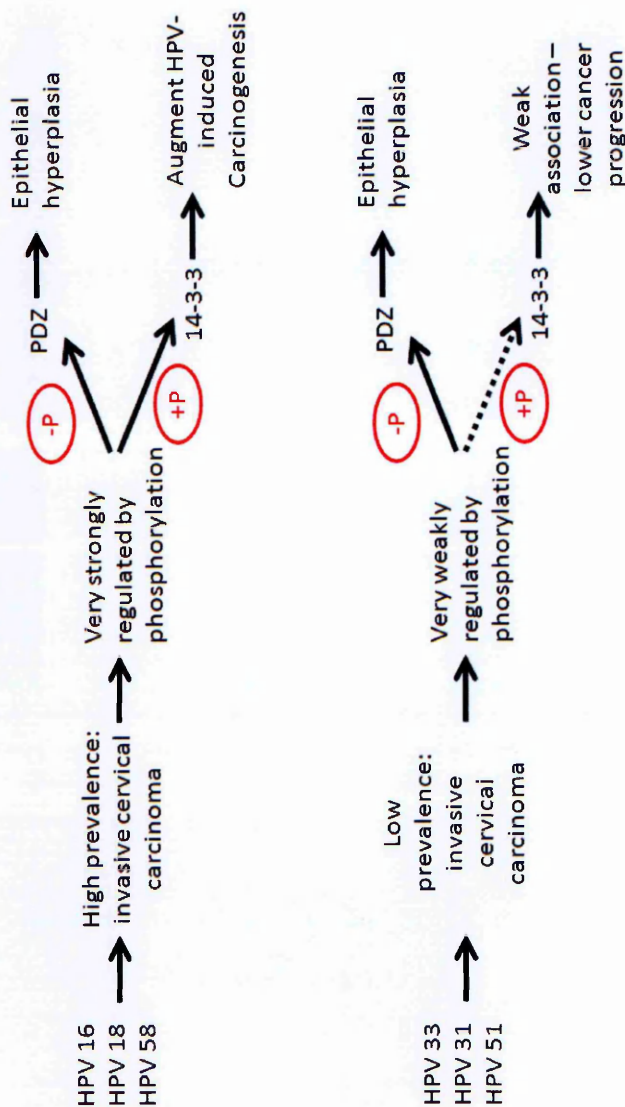
than HPV-18 *in vivo*. As for HPV-31 E6, it displays a quite different susceptibility to phospho-regulation from HPV-18 and -16 E6. Whether these different potentials in phosphorylation have an impact on this virus's fitness remains to be determined. But these differences should be taken into consideration when applying data obtained from the study of one HPV type to the life cycle of another HPV type.

In the case of tumour involvement, HPV-16, -18 and -58 have generally higher association with invasive cervical carcinoma, followed by HPV-33, -31 and -51 (Li *et al*, 2011a). Among these HPV types, HPV-16 and -18 remain the leading causative agents for cervical cancers, and are followed by HPV-58 which has been reported to be of increasing clinical importance, especially in Eastern Asia (Li *et al*, 2011a; Chan, 2012; Aromseree *et al*, 2014). Susceptibility to phosphorylation may be a means for the virus to survive in the epithelial cells derived from different tissues. For instance, HPV-16, HPV-58 and HPV-31, which belong to the genus Alpha-9, associate more commonly with squamous cell carcinoma (SCC) than with adenocarcinoma (ADC), whilst HPV-18, which belongs to genus Alpha-7, is commonly found in ADC (Clifford *et al*, 2003b; Smith *et al*, 2007; Li *et al*, 2011a). SCC is found at the ectocervix and arises from squamous cells which are continuously sloughed off and replaced, whilst ADC arises from glandular cells at the endocervix. There are different gene profiles in the squamous and glandular normal cervical cells, and these include genes encoding epidermal growth factor receptor (EGFR), PI3K and K-ras. The expression of these genes is also different when these normal cells become transformed to SCC and ADC, respectively. For instance, the EGFR gene is

expressed in the basal and parabasal layers of the ectocervical normal epithelial cells, whilst expression of this gene is not detected in glandular cells (Kupryjańczyk, 1990). The gene encoding PI3K is expressed at a low levels in normal cervical epithelial cells (Yao *et al*, 2008). Activation of EGFR can subsequently lead to activation of PI3K, and this is important for cell cycle progression, growth and proliferation (Prenzel *et al*, 2001). The gene encoding EGFR is often found mutated and overexpressed in SCC (Wright *et al*, 2013; Maiti *et al*, 2013), whilst the PI3K catalytic domain is more frequently mutated in SCC than in ADC (Wright *et al*, 2013). These mutations result in the upregulation of survival kinases, including AKT, MAPK and ERK signalling pathways (Davis *et al*, 2014). Normally, K-ras expression is kept at a low level in cervical cells (Agnantis *et al*, 1988; Sagae *et al*, 1989), where it is important in cell cycle regulation, apoptosis and Raf-1 signalling (Jancik *et al*, 2010). K-ras can be activated by point mutations, and high level of K-ras expression has been implicated in many cancers, including lung, ovarian (Slebos *et al*, 1991; Jancik *et al*, 2010; Yoo *et al*, 2012; Alomari *et al*, 2014) and also in ADC (Wright *et al*, 2013; Matsubara *et al*, 2014). K-Ras mutation can lead to an increase the abundancy of 3', 5'-cyclic adenosine monophosphate (cAMP) which subsequently activates PKA (Konishi-Imamura *et al*, 1987; Franks *et al*, 1987; Konishi-Imamura *et al*, 1988), as well as activating the MAPK and PI3K pathways (Affolter *et al*, 2013; Chang *et al*, 2014). Therefore, it is intriguing to speculate that differences in the signalling pathways in the two different cervical tissues might contribute towards the preference for specific viral types in each location. Obviously, the involvement of E6 in

regulating these pathways might contribute towards this, although future studies will be required to investigating this further.

It is also interesting to speculate about the other HR HPV types, such as HPV-35, -39, -45, -52, -56 and -59. Besides PKA and AKT, other kinases, such as tyrosine kinases (Lck and Src kinases) and Cdc-2-like kinase, Clk2, which are also serine/threonine kinases, might be involved in phosphorylating other HR HPV types E6s as predicted and these are shown in the Appendix. The differential susceptibility to phosphorylation across the different HPV E6s could be reflected in the consequences upon E6 recognition of PDZ-containing proteins and 14-3-3. Indeed, this was found to be very similar across the different E6 oncoproteins, with phosphorylation of the PBM, even if only weak, nonetheless leading to a reduction in PDZ binding potential. All these differential phospho-regulation reactions of HPV E6 proteins and their correlation to PDZ or 14-3-3 recognition, with the possible biological implications are depicted in Figure 32.



**Figure 32. The prevalence of HPV types in invasive cervical carcinoma contributed by phosphorylation and consequent recognition of PDZ-containing proteins or 14-3-3.** The high risk HPV types detected in invasive carcinoma are ranked in order of decreasing prevalence: HPV-16, -18, -58, -33, -31 and -51 globally (Li *et al*, 2011a). The HPV-16, -18 and -58 E6 oncoproteins have a higher prevalence in invasive cervical carcinoma and are highly regulated by the survival kinases PKA and/or AKT; whilst HPV-33, -31 and -51 are correlated with invasive cervical carcinoma to a lesser extent and they are moderately or very weakly regulated by PKA or AKT. In this case, phosphorylation inhibits the ability of E6 to recognise PDZ-containing proteins, conferring recognition of 14-3-3 proteins, many of the whose isoforms possess oncogenic potential. Thus, high levels of phosphorylation enhance the association with 14-3-3 proteins, and may contribute to HPV-induced carcinogenesis. -P and +P indicate the conditions without and with phosphorylation respectively.



## 4.2 Consequence of E6 phosphorylation

### 4.2.1 Phospho-status of E6 determines PDZ and 14-3-3 recognition

It has been known for some time that phosphorylation of HPV-18 E6 could inhibit PDZ binding activities (Kühne *et al*, 2000; Zhang *et al*, 2007), but there was no data on whether other HR HPV E6 proteins would behave in a similar manner. In a series of comparative assays, it was found that phosphorylation within the E6-PBM inhibited PDZ interactions irrespective of the kinase or HPV type. Intriguingly, by analysing the amino acid sequence of HPV-31 E6, I found that there are two potential phospho-acceptor sites within the last six amino acids of its carboxy terminus. This protein is a substrate for both AKT and PKA, but AKT would appear to phosphorylate T147 within the PBM, whilst PKA phosphorylates HPV-31 E6 at position S82, which is well away from the PBM. This suggests that HPV-31 E6 will be regulated very differently from the other HPV E6 oncoproteins analysed, including its PDZ recognition. Thus, in the presence of PKA activity, PDZ recognition by HPV-31 E6 will be unaffected, since the PBM will be left unmodified. It is interesting to notice that in comparison with HPV-16, -51 and -58 E6, HPV-31 E6 contains an Arginine (R) residue at -2 from S82 and this can fit into a PKA recognition motif. From crystal structure studies by Nominé *et al*, it was shown that the S82 residue is exposed and is located within the interdomain which connects both of the zinc finger domains of E6 (Ristriani *et al*, 2002; Nominé *et al*, 2006), the region through which E6 associates with p53, E6AP and CBP/p300. This indicates that S82 can be accessed by PKA and that phosphorylation may confer

a conformational change on E6, subsequently affecting its ability to recognise these proteins. However, this possibility remains to be investigated and understanding this aspect is important, as it may have an impact on the pathogenicity of HPV-31, as well as other HPV types that could potentially be regulated in a similar manner.

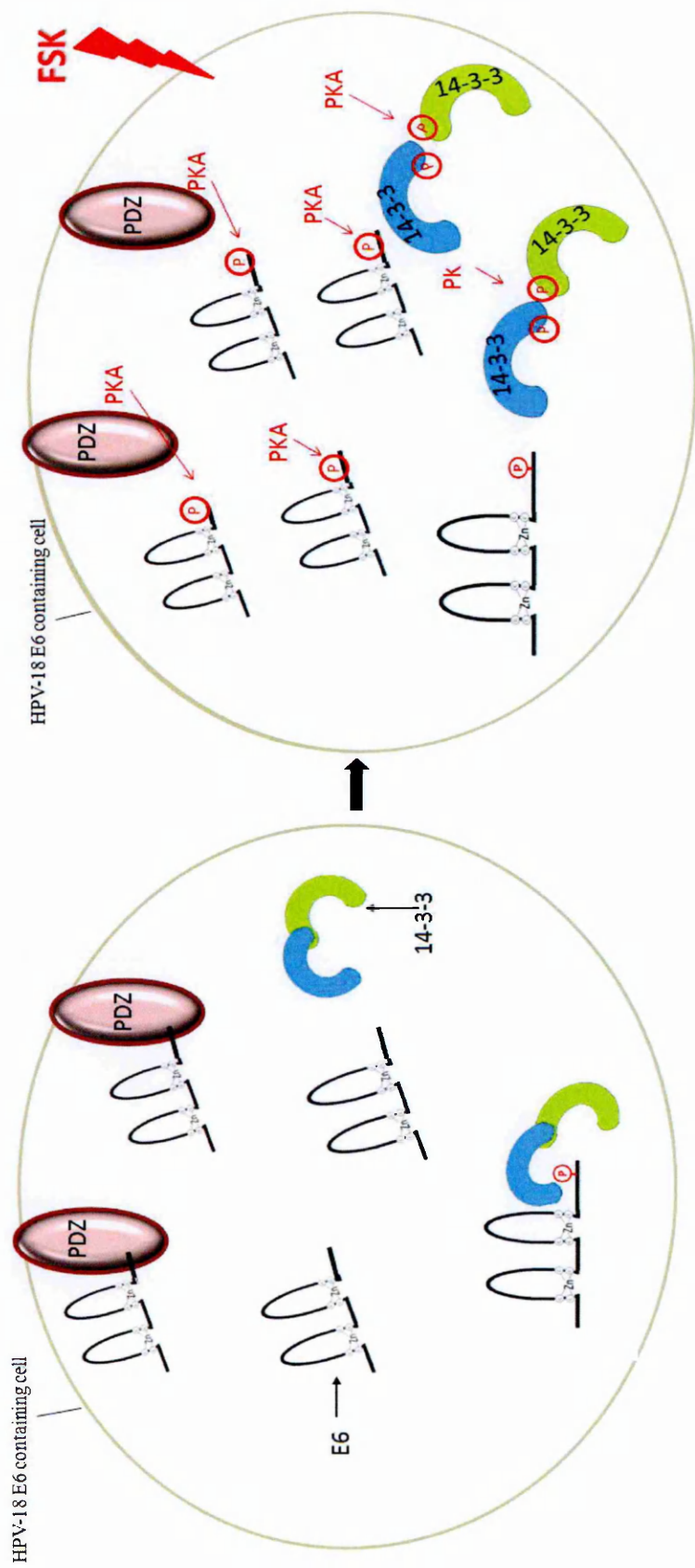
This study shows clearly that phosphorylation of E6 also confers the ability to interact with a new class of cellular targets, 14-3-3 proteins, which have been defined as phosphoserine/threonine binding proteins (Muslin *et al*, 1996). This indicates that phospho-regulation of E6 will be critical in controlling the precise function of this important region of the E6 oncoprotein. A series of studies were performed to compare the relative efficiencies, and subsequent consequences, of the phosphorylation of HPV E6 oncoproteins derived from a panel of different cancer-causing HPV types. Most importantly, it was found that most of the HR, but not LR, HPV E6 proteins analysed showed phosphorylation-dependent interactions with different 14-3-3 family members. The strength of interaction was a direct reflection of the efficiency with which the different E6 oncoprotein PBMs were phosphorylated by the different kinases. It was also interesting to note that whilst there were some subtle differences in how strongly the different HPV E6 proteins recognised different 14-3-3 family members, the general trend indicated that  $\zeta$ ,  $\epsilon$  and  $\gamma$  are the most commonly bound. However, an exception applies to the 14-3-3 $\beta$ , which associates with both LR and HR HPV E6 independently of phosphorylation. Interestingly, even though HPV-31 E6 can be phosphorylated by PKA at S82, phosphorylation at

this site does not confer 14-3-3 interaction, showing that except for 14-3-3 $\beta$ , the 14-3-3 proteins interact with E6s at their PBMs, which in the case of HPV-31 E6, is largely dependent on AKT phosphorylation. All of these assays were done *in vitro*, obviously, the *in vivo* situation can be more complex. However, co-immunoprecipitation with HPV-18 and -16 E6 in fact revealed similar patterns of interaction, with the 14-3-3 $\zeta$  and  $\theta$  isoforms being predominant, whilst  $\epsilon$  associates well with HPV-18 E6 but less so with HPV-16 E6. Consistently, *in vivo* assays also showed that the association is also dependent upon the phospho-status of E6 and the integrity of the E6-PBM.

I also investigated whether the potential interactions of HR HPV-33, -51 and -58 E6 with 14-3-3 $\epsilon$  reflect their respective susceptibility to phosphorylation. I found that HPV-18 E6 mimicking HPV-51 E6 protein, appear to be a poor substrate for both PKA and AKT, and does not interact with 14-3-3 $\epsilon$ . As for HPV-58 E6, PKA and AKT phosphorylation confers a strong 14-3-3 $\epsilon$  association. This observations further highlights the finding that different HR HPV types E6s may have distinct preferences for targeting different subsets of cellular proteins. For instance, it is well-known that HPV-16 E6 preferentially targets Scribble whilst HPV-18 E6 preferentially targets hDlg (Thomas *et al*, 2005), although both of these proteins belong to the same Scribble polarity module (Humbert *et al*, 2008). On top of this is the differential regulation by kinases which also has an important influence on their choice of target proteins.

In order to establish whether the interaction between 14-3-3 and E6 could be enhanced, Fsk, a synthetic PKA activator was added to the cells. Fsk can induce

at least a 3-fold increase in PKA activity (Ekholm *et al*, 1997; Ahn *et al*, 2007; Cyphert *et al*, 2014), by activating cAMP through adenylyl cyclase (Seamon *et al*, 1981; Takeda *et al*, 1983). In theory, Fsk stimulation should increase PKA-phosphorylation of E6 and, hence, increase interaction with 14-3-3. However, the opposite outcome was observed where Fsk stimulation reduced the E6 interaction with 14-3-3. This is most likely explained by the fact that very high levels of PKA activity will also result in 14-3-3 phosphorylation, which subsequently inhibits the dimerisation and binding activities of 14-3-3 (Gu *et al*, 2006), as illustrated in Figure 33. Therefore, the use of synthetic activators or inhibitors in this analysis is not appropriate in studying the biological consequences of the E6-14-3-3 association. Even though these synthetic kinase activators or inhibitors have been widely used to study the kinase activity in cells, the drawbacks are their lack of specificity in activating or inhibiting the downstream signalling cascades. Besides activating PKA, Fsk-induced activation of cAMP can also activate AKT and ERK (Filippa *et al*, 1999; Gao *et al*, 1999; Schmitt & Stork, 2000), or inhibit MAPK (Siddhanti *et al*, 1995; Plevin *et al*, 1997). As for H89, it inhibits PKA efficiently but also inhibits S6K1 and MSK1 as efficiently, and can also inhibit AKT and MAPK to a lesser extent (Davies *et al*, 2000).



**Figure 33. Forskolin inhibits interaction between phospho-E6 and 14-3-3.** In HPV containing cells, HPV-18 E6 interacts with PDZ-containing proteins, such as hDlg and Scribble. A minority of HPV-18 E6 is phosphorylated and interacts with 14-3-3. When the cell is stimulated with Forskolin (Fsk), this enhances PKA activity in the cells by at least 3-fold. In this case, most of the HPV-18 E6 will be phosphorylated by PKA. However, this does not confer interaction with 14-3-3, as PKA phosphorylation affects 14-3-3 dimer formation, and 14-3-3 monomers do not bind to phospho-E6.

Although Fsk inhibits E6-14-3-3 association, this might actually be a useful way of looking for other potential phosphorylation-dependent interactions of HPV E6. For example, Forkhead-associated (FHA) domains-containing proteins (Durocher & Jackson, 2002; Hammet *et al*, 2003) and MH2 (MAD Homology 2) domain containing proteins (Wu *et al*, 2001), and several others (Table 4), are all phosphoserine/threonine binding proteins, and proteomic analyses would be one possible approach to study this further.

**Table 4: Example of phosphoserine/threonine domain containing proteins.** WW domains, Forkhead associated (FHA) domains, WD40 or Leucine rich repeat (LRR) domains, with their respective phospho-binding motifs and binding partners. Adapted from Yaffe & Elia (2001).

Protein	Consensus motif	Binding partner	References
<b><u>WW domain</u></b> a) Pin1 b) NEDD4	pSP or pTP	Cdc25C, Tau, Wee1, Plk1, Cdc27	Lu <i>et al.</i> , 1999; Elia <i>et al.</i> , 2003
<b><u>FHA domain</u></b> a) Rad53 b) KAPP c) Nipp1 d) Chk2	pT	Rad9 Receptor-like kinases Cdc5L	(Durocher & Jackson, 2002)
<b><u>WD40/LRR domain of F-box proteins</u></b> a) Cdc4 b) Pop1/2 c) Grr1 d) Skp2	L-I/L/P-pT-P DpSGXXpS	Sic1, Far1, Cdc6 Cdc18, Rum1 Cln1/2 p27	Orlicky <i>et al.</i> , 2003

In some cases, 14-3-3 can recognise proteins in which phosphorylation is replaced by a phospho-mimic mutation, such as Aspartic (D) or Glutamic acid (E), and in some cases it does not. In the cases of Bassoon protein (a presynaptic anchoring scaffold protein) (Schröder *et al*, 2013), or IRSp53 (insulin receptor substrate p53/58) (Cohen *et al*, 2011), phospho-mimic mutants do not bind to 14-3-3, whereas a phosphomimic mutant of YAP (Yes-associated protein) showed a reduced ability to interact with 14-3-3 compared with the wild type YAP (Zhao *et al*, 2010). In contrast, a phosphomimic mutant of Bruton tyrosine kinase (Btk) interacts strongly with 14-3-3 (Mohammad *et al*, 2013). In the case of HPV-18 E6, substituting Thr156 with an acidic residue does not result in a phospho-mimic with respect to interaction with 14-3-3 $\zeta$ , even though the HPV-18 E6 T156E mutant is weakly recognised by the anti-phospho-E6 specific antibody. This indicates that in the case of HPV-18 E6, 14-3-3 $\zeta$  recognition is phosphate-dependent, and is not amenable to simple charge substitution. This result also has one other very important consequence. Thus, simple deletion, or even single point mutational ablation of the Ser/Thr residue embedded within the PBMs of the high risk HPV E6 oncoproteins inhibits PDZ association, as well as abolishing interaction with 14-3-3. It is therefore extremely important that this is taken into consideration when assigning biological functions to this particular region of the E6 oncoprotein.

All of the above regulation by phosphorylation is observed only in HR HPV E6 proteins. Due to the lack of the PBM in LR HPV E6, such regulation by PKA is absent, and the same will be expected for AKT. However, this does not exclude

the possibility that LR HPV E6s can be regulated by kinases such as PKC or ATM kinase (Appendix). Having said that, there is a smaller number of kinases predicted to phosphorylate LR HPV E6 proteins from the scansite. This may serve as a hint that phospho-regulation or other post-translational modifications may not be a common feature of the LR HPV types E6 which may be one of the reasons for their weaker oncogenic potential, compared with the HR HPV types. However, it is possible that if the LR HPV E6s can be phosphorylated at a serine/threonine residue, this may confer interaction with a phosphoserine/threonine binding protein and these possibilities require further investigation.

#### 4.2.2 Phosphorylation stabilises E6

Previous studies had indicated that an intact E6 PBM might have an effect on E6 stability (Nicolaidis *et al*, 2011), and I wondered if this might be related to the ability of E6 to be phosphorylated. To investigate this, a series of analyses were done to assess the stability of the phospho-E6 protein. The results demonstrated that phosphorylated HPV-18 E6 has a much longer half life than the non-phosphorylated form of the protein. Furthermore, it is also intriguing to note that E6\* appeared to be more stable under conditions when the full-length E6 protein was phosphorylated. Since E6\* lacks the PBM/phospho-acceptor site, this suggests that its increased half life may be mediated, in part, through complex formation with the full length phosphorylated E6 oncoprotein. Further studies are obviously required to confirm this.



Certainly, the increased stability of phosphorylated forms of HPV E6 protein might be aided by the association with 14-3-3. It has been reported previously, that 14-3-3 association can modulate the stability of its binding protein, such as for Bad (Chiang *et al*, 2001) and tristetrapolin (TTP) (Sun *et al*, 2007; Kraemer & Clement, 2014). In the case of HPV-18, loss of 14-3-3 expression in HeLa cells does reduce the steady state levels of E6, suggesting that phosphorylated E6 can be stabilised by 14-3-3 interaction. However, it is important to note that the extension of E6 half life following Fsk treatment is most likely independent of 14-3-3 interaction, since under these conditions, the E6-14-3-3 interaction is disrupted. Possible explanations for this include HPV E6 interaction with other phosphoserine/threonine proteins, some of which are listed in Table 4. Alternatively, Fsk treatment itself is likely to increase protein synthesis (Xenophontos *et al*, 1989), this may include increased level of E6AP and EDD, which in turn, associate with E6 and contribute to E6 stability independent of E6s phospho-status.

#### 4.2.3 Cell cycle regulation of E6 phosphorylation and E6's activities during S and G2/M phases

Analysis of the HPV-16 and -18 E6 phospho-status *in vivo* also confirmed important differences in how the two proteins are phosphorylated. In asynchronously growing cells, HPV-16 E6 was consistently phosphorylated at a higher level than HPV-18 E6, probably reflecting the high levels of AKT

activity under these conditions. However, following Fsk treatment, HPV-18 E6 was most strongly phosphorylated, and this increase was not as apparent for HPV-16 E6. This is consistent with the *in vitro* analyses showing HPV-18 E6 to be a better substrate of PKA than HPV-16 E6.

Based on this, I then proceeded to investigate whether E6 would be differentially phosphorylated through the cell cycle, since this is a more physiologically relevant way of analysing the phospho-regulation of HPV E6. Indeed, the differences in phosphorylation are even more apparent in the cell cycle analyses, which showed that HPV-18 E6 is phosphorylated most strongly during G2/M. Interestingly, the total levels of E6 appear lower during G1/S and S phases and this might be a reflection of lower levels of E6 phosphorylation under the influence of Aphidicolin, although this is not apparent when the cells were synchronised using a double thymidine block. The mechanism of actions of Aphidicolin and the double thymidine block are different. Aphidicolin inhibits DNA replication through DNA polymerase and can affect the cell growth (Spadari *et al*, 1982), whilst high concentration of thymidine in the cells can synchronise the DNA synthesis in S phase in a more physiological way. However, this is perhaps less robust than an Aphidicolin block, as DNA synthesis can be continued at a low level (Bostock *et al*, 1971). Interestingly, the highest levels of phosphorylation and E6 stability coincide with G2/M, which has also been reported previously to be when PKA activity is at its highest (Daub *et al*, 2008; Chandrasekhar *et al*, 2013). A recent study showed that AKT activity is the highest 4 hrs post-release from a double thymidine

block when the cells are in S phase (Liu *et al*, 2014). Consistent with this, HPV-16 E6 is most heavily phosphorylated during S phase, and this is also reflected in the levels of its expression. Taken together, these analyses reflect very clearly the discrete phases of the cell cycle when the HPV-16 E6 and HPV-18 E6 oncoproteins are most heavily phosphorylated. Although the other HR HPV E6 proteins were not analysed *in vivo*, one would predict that HPV-58 E6 would probably be heavily phosphorylated throughout the cell cycle, being a very good substrate for both AKT and PKA. As for HPV-31 E6, it would have a PBM phosphorylation pattern very similar to that of HPV-16 E6, but will instead have a S82 phosphorylation in the G2/M phase of the cell cycle.

Cell cycle analyses showed compelling evidence linking *in vivo* phosphorylation of E6 with its increased association with 14-3-3 proteins and Cdc25C, potentially as a complex. In the case of HPV-18 E6, these interactions were always strongest during G2/M which corresponds to the phase in which HPV-18 E6 is most heavily phosphorylated. In the case of HPV-16 E6, interaction with 14-3-3 and Cdc25C proteins was highest during S phase, and again, this corresponds to the phase of cell cycle when HPV-16 E6 is most strongly phosphorylated. These differences may indicate that 14-3-3 may contribute to the stability of phospho-E6 during the different stages of the cell cycle, with the stability of HPV-16 E6 being maintained throughout the epithelial layers and HPV-18 E6 in the upper epithelial layer. This also implies that HPV-16 E6 interacts with 14-3-3 more intensively than HPV-18 E6. In addition, 14-3-3 functions might also be perturbed differently at the different

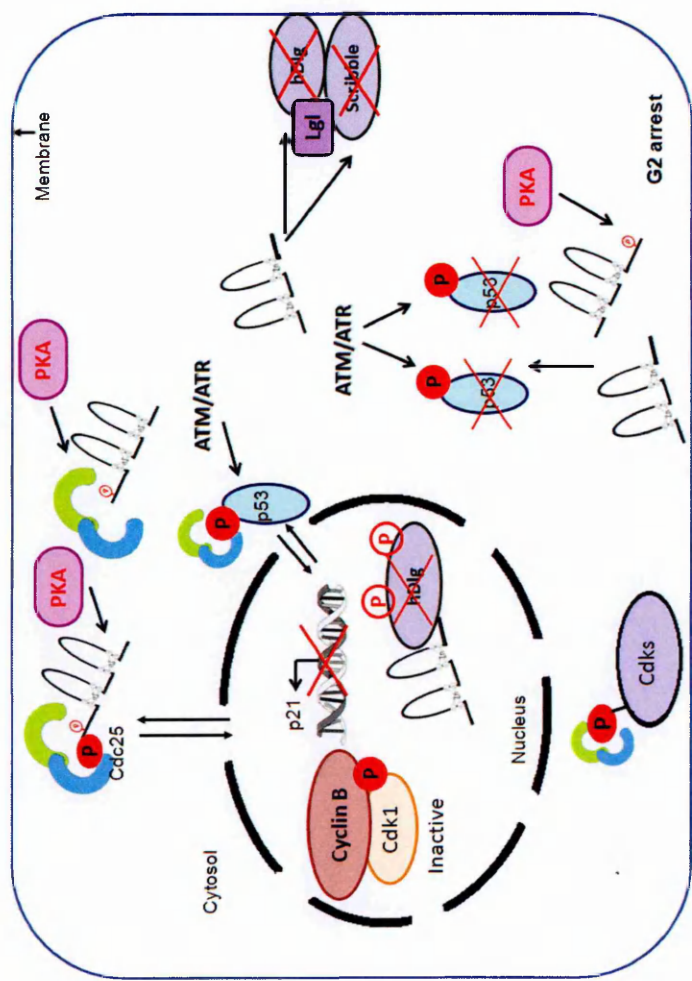
epithelial layers during the viral life cycle. In comparison, interactions with E6AP were found to be relatively unchanged throughout the cell cycle. Another intriguing feature of these studies was the behavior of the E6 oncoproteins with respect to hDlg and Scribble. Based on the *in vitro* analyses, one would predict that E6-Dlg interactions would decrease during G2/M for HPV-18 E6 and S phase for HPV-16 E6, when the E6 is most heavily phosphorylated. However, this is not the case, and a clear increase in the interaction of both HPV-18 and -16 E6 proteins with hDlg occurs during G2/M. The most obvious explanation for this is that post-translational modifications to hDlg actually increase its ability to interact with a subset of unmodified E6 at this period of the cell cycle. Indeed, the result shown in Figure 29(A) and consistent with previous studies, suggesting that nuclear forms of phosphorylated hDlg were more readily targeted by the E6 oncoprotein (Narayan *et al*, 2009b; Massimi *et al*, 2006), and these studies go some way to support those observations. Likewise, HPV-18 E6 also interacts most strongly with Scribble during G2/M, whilst HPV-16 E6 interaction with Scribble is strongest during S phase and reduces somewhat during G2/M phase. The reason for these differences might again partially reflect differences in the phospho-status or localisation of the target protein, but they are also most likely a direct reflection of changes in the total amount of E6 present during these different phases of the cell cycle. Obviously, a major question for future studies is to determine the percentage of E6 that is phosphorylated at any given time and, furthermore, whether there is any significant dephosphorylation of E6 at different cell cycle phases.

Previous studies showed that HPV E6 and E7 are involved in perturbing cell cycle checkpoints and this is partly the result of the abrogation of p53 and pRB functions by E6 and E7, respectively (Demers *et al*, 1994; Wazer *et al*, 1995; Stewart *et al*, 1995; Xiong *et al*, 1996; Dyson, 1998). The p53 protein accumulated at cell cycle checkpoints (Darzynkiewicz *et al*, 1986), and the majority of p53 is degraded by E6 efficiently. However, there is detectable levels of p53 in HeLa cells, and the levels of p53 increase from G1 to S phase. This could be due to the low levels of E6 during these phases of the cell cycle, and hence, the levels of p53 protein does not change significantly, as this pattern of p53 protein levels is similarly to that observed in the previous studies performed using HPV negative cells (Reich & Levine, 1984; Danova *et al*, 1990; Gudas *et al*, 1994). Intriguingly, I also found that, there is a dramatic loss of p53 in G2/M phase of the cell cycle, especially its cytoplasmic pool (Figure 29). At this phase, p53 is probably phosphorylated, which consequently activates the G2/M checkpoint (Dellinger *et al*, 2003), and this is partly overcome by E6 degrading p53. Ablation of HPV-18 E6 using siRNA against HPV-18 E6 and E7 restores the levels and correct expression pattern of p53, with higher levels of nuclear than cytoplasmic forms of p53. Taken together, the results in this study show that HPV-18 E6 appears most active and exerts its multiple functions during G2/M, including degrading p53 and PDZ-containing proteins, and deregulating cell cycle, partly through 14-3-3 and other 14-3-3 functions. In the case of HPV-16 E6, these activities are also very apparent during S phase and continue into G2/M. The involvement of HPV E6s in

regulating the cell cycle events, both within an individual cell and more globally, are depicted in Figure 34 and 35, respectively.

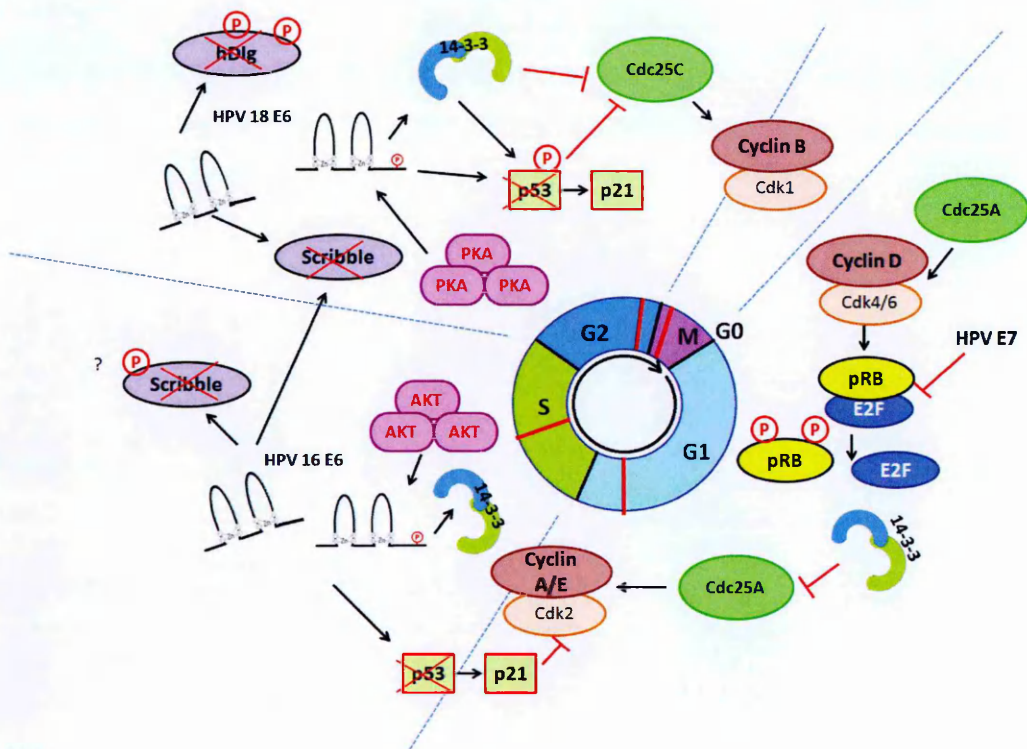
#### 4.2.4 E6 perturbs the subcellular localisation of 14-3-3 and p53 transcriptional transactivation.

As mentioned earlier, most of the 14-3-3 isoforms, with the exception of  $\sigma$ , are overexpressed and possess oncogenic potential in cancers (Lodygin & Hermeking, 2005; Danes *et al*, 2008; Bai *et al*, 2014; Zhao *et al*, 2014). One of the major functions of 14-3-3 proteins is to regulate the subcellular distribution of their different binding partners, and this is linked very closely to the correct spatial distribution of 14-3-3 between the nucleus and cytoplasmic fractions of the cell (Brunet *et al*, 2002; reviewed in Muslin & Xing, 2000; Seimiya *et al*, 2000). In most cases, 14-3-3 interacts with phospho-proteins and keeps them in an inactive form in the cytoplasm, as for Raf-1 (Wan *et al*, 2004; Ritt *et al*, 2010; Radhakrishnan & Martinez, 2010), Bad (Rommel *et al*, 1997; Dumaz & Marais, 2003) and Cdc25 (Peng *et al*, 1997; Esmenjaud-Mailhat *et al*, 2007; Chan *et al*, 2011). Nuclear 14-3-3 was suggested to be either unbound 14-3-3 or to be associated with transcriptional co-factors in the nucleus, such as the Forkhead transcription factors (Brunet *et al*, 2002; Obsilova *et al*, 2005) and p53 (Rajagopalan *et al*, 2010). In addition, 14-3-3 is also found to relocate in the nucleus during mitosis (Su *et al*, 2001). In cancer cells, 14-3-3 is overexpressed and the normal distribution and functions of these proteins



**Figure 34. Function of HPV-18 E6 during G2/M phase within an individual cell.** During G2/M phase, the increased level of PKA phosphorylates most of the pool of E6 and this increases the steady state levels of E6. This confers increased interaction with 14-3-3, resulting in 14-3-3 retention in the cytosol. 14-3-3 normally shuttles Cdc25C and p53 in and out of the nucleus. Retention of 14-3-3 results in retention of both Cdc25C and p53 in the cytosol, thereby inhibiting the activation of Cdk2/CyclinB complex and p21 transcription transactivation, mediated by Cdc25C and p53, respectively. Simultaneously, the tumour suppressors and the cell polarity complex are downregulated by E6: activation of p53 by ATM/ATR kinases also results in the degradation of p53 by E6; the components of the Scribble polarity module, hDlg and Scribble, associate with the E6 PBM, resulting in the degradation of hDlg and Scribble.





**Figure 35. Involvement of HPV oncoproteins in the different phases of cell cycle.** During G1 phase, Cdc25A can dephosphorylate and activate the Cyclin D/Cdk4/6 complex. This, in turn, allows Cyclin D to phosphorylate pRB which bound to E2F. Hyperphosphorylated pRB dissociates from E2F, allowing cells to progress to S phase. The presence of HPV E7 targets and degrades hypophosphorylated pRB, this also allows cells to progress to S phase. At G1/S, Cdc25A can activate the Cyclin A/E/Cdk2 complex and allow cells to progress to S phase. However, during the normal cell cycle checkpoint, or when cells sustain DNA damage, Cdc25A is phosphorylated and this creates a 14-3-3 binding motif. 14-3-3 binds and keeps Cdc25A inactive in the cytosol. When cells progress to S phase, the levels of AKT increase and this could also be enhanced by the presence of HPV E7. This results in the phosphorylation of HPV-16 E6, allowing association with 14-3-3 and Scribble. In G2/M phase, the levels of PKA increase and phosphorylate HPV-18 E6, also resulting in association with 14-3-3 and retention of 14-3-3 in the cytosol. HPV-16 E6 can also be phosphorylated at this phase, although this seems to occur at a lower level compared with HPV-16 E6 phosphorylation during S phase. Both HPV E6s associate with hDlg and Scribble through E6-PBM-PDZ recognition, resulting in the degradation of hDlg and Scribble. Meanwhile, p53 plays a crucial role in cell cycle checkpoints by activating p21, which then inhibits Cyclin-dependent kinases (Cdk1 and Cdk2). However, this is abrogated when the majority of p53 is degraded by HPV E6. The remaining pool of p53, which are phosphorylated can bind to 14-3-3 and this keeps p53 away from p21 transcriptional transactivation in the nucleus. Similarly, 14-3-3 associates with phosphorylated Cdc25C and keeps it as an inactive form in the cytosol.



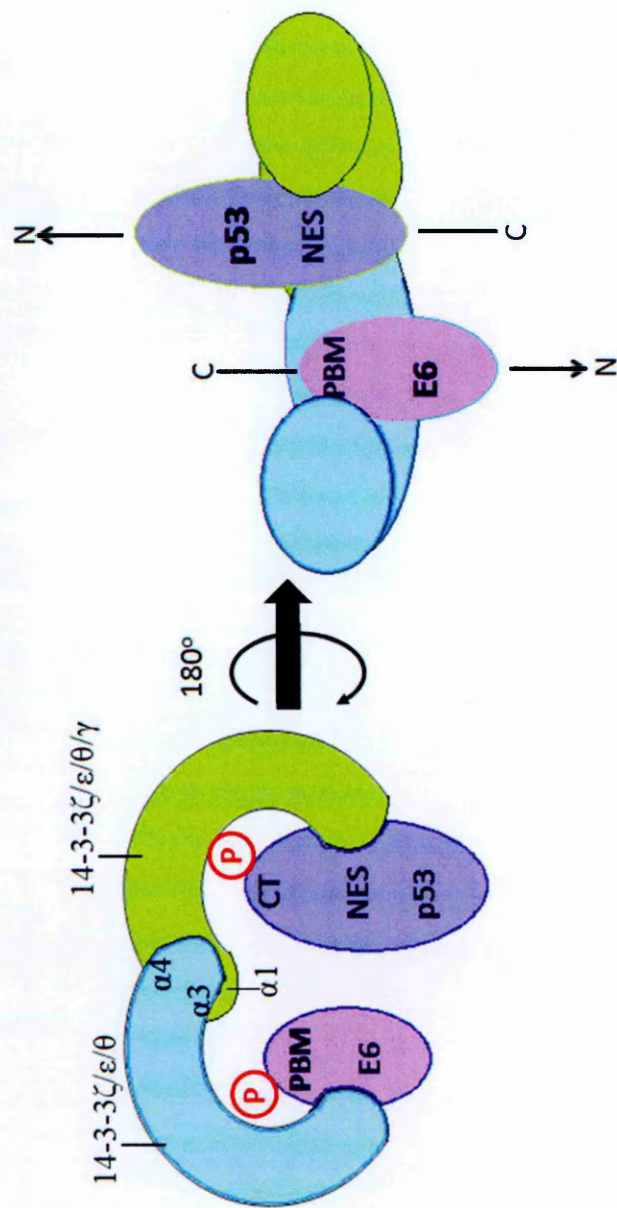
regulated by 14-3-3 can be perturbed. As a consequence, the cell survival signals, such as Ras-Raf-MAPK pathways can be over-activated, and cell cycle progression can be dysregulated, for example through abrogation of p53 and Cdc25 function. To investigate whether E6 might perturb 14-3-3 distribution, cell fractionation analyses were done in G2/M phase cells, where E6 associates with 14-3-3 most strongly. In G2/M phase cells, in the presence of E6, 14-3-3 was localised mainly in the cytosol. However, when E6 is not interacting with 14-3-3, either through loss of E6 or loss of its PBM, the nuclear localisation of 14-3-3 was restored. Taken together, these results indicate that the consequence of the E6-14-3-3 association is a reduction in the level of nuclear 14-3-3, and hence potentially a perturbation of 14-3-3 mediated functions in the nucleus.

As mentioned earlier, 14-3-3 is involved in controlling the shuttling of proteins in and out of the nucleus (Brunet *et al*, 2002). Two prime candidates to be affected by E6-induced alteration in the subcellular distribution of 14-3-3 are Cdc25 and p53, which are the two important components of G2/M checkpoint controls. The results in Figure 29 showed that the majority of Cdc25C remains in the cytosol, and the phospho-Cdc25C which also remains in the cytosol during G2/M phase. The downregulation of HPV-18 E6 leads to reduced levels of Cdc25C. This is due to the upregulation of p53 following ablation of HPV-18 E6, which is in agreement with the notion that the downregulation of Cdc25C is a means to induce a G2 arrest, which is tightly regulated by p53 (St Clair *et al*, 2004; St. Clair & Manfredi, 2006). However, Cdc25C is overexpressed in many cancers, which results in an imbalance in normal cell

cycle regulation (Boutros *et al*, 2007). Therefore, the presence of E6, which downregulates p53, allows high levels of Cdc25, possibly resulting in genome instability in cancer cells (Bugler *et al*, 2006; Ray *et al*, 2007; Dalvai *et al*, 2013).

Previous studies showed that 14-3-3 interacts with p53 in a phosphorylation-dependent manner (Agarwal *et al*, 1995; Waterman *et al*, 1998). Binding of 14-3-3 at the C-terminal region of p53 increases p53's ability to bind to DNA and this enables p53 to activate its downstream transcriptional targets such as p21 (Hupp *et al*, 1992; Halazonetis *et al*, 1993; Agarwal *et al*, 1995; Waterman *et al*, 1995, 1998; Rajagopalan *et al*, 2008). I was therefore interested in investigating any potential function for residual pools of p53 in HeLa cells, and the results showed that 14-3-3 interacts with p53 especially during the G2/M phase. Downregulation of HPV-18 E6 is thought to increase the levels of p53 and this should increase the p53-14-3-3 interaction. However, this does not seem to be the case, as can be seen in Figure 30B, HeLa cells transfected with siRNA against HPV-18 E6/E7, despite showing increased levels p53 do not show an increase in the amount bound to 14-3-3. This indicates that E6 does not abrogate the 14-3-3 association with p53. Therefore, an alternative possibility was that E6 might alter the function of the 14-3-3-p53 complex and I reasoned that cytoplasmic retention of 14-3-3 during G2/M might impair p53 transcriptional transactivation function. Indeed, in a series of transfection assays, I found that in the absence of p53 degradation E6 could still effectively inhibit p53 transcriptional activation of the p21 promoter. This activity of E6

was found to require not only an intact PBM, but most importantly also an intact phospho-regulation site upstream of T156, suggesting that the ability to bind 14-3-3 is important for E6's ability to inhibit p53 transcriptional activity. Since the HPV-18 E6 R153A mutant cannot be phosphorylated by AKT or PKA even though its PBM remains intact, and hence it does not associate with 14-3-3, this mutant will also be very useful to address the existence of 14-3-3-p53-E6 complex, the role of E6 in perturbing 14-3-3 nuclear localisation and the p53 transcriptional transactivation mediated by 14-3-3 during G2/M phase. It is expected that this mutant should not have an effect on 14-3-3 localisation, similar to the  $\Delta$ PBM mutant. E6 and p53 potentially fit into the 14-3-3 binding pocket in an anti-parallel manner, as illustrated in Figure 36, hence, this may not result in p53 degradation by E6. This probably occurs through retention of 14-3-3 in the cytosol, and obviously future studies will be required to determine whether p53 is also present in this complex. This can be done by performing sequential co-immunoprecipitation and immunofluorescence. H1299 cells can be transiently transfected with HA-tagged HPV-18 E6 and Flag-tagged p53 expression plasmids. The HA-18 E6 can be immunoprecipitated using anti-HA conjugated agarose beads, and the bound complex is further immunoprecipitated using anti-Flag conjugated agarose beads. Subsequently, western blot analysis can be performed to detect the bound endogenous 14-3-3. As controls, HPV-18 E6  $\Delta$ PBM or R153A mutants can be included. Alternatively, immunofluorescence assays can be performed to detect the colocalisation of 14-3-3, p53 and E6 proteins. A particularly exciting aspect to



**Figure 36. Schematic diagram of 14-3-3-E6-p53 complex formation.** 14-3-3 $\zeta$ ,  $\epsilon$ ,  $\theta$  and  $\gamma$  dimer can bridge the association of E6 and p53. This occurs through binding of 14-3-3 $\zeta$ ,  $\epsilon$  or  $\theta$  to the phosphorylated (red P) C-terminal PDZ binding motif (PBM) of HPV-E6, whilst 14-3-3 $\zeta$ ,  $\epsilon$ ,  $\theta$  or  $\gamma$  bind to the C-terminus of phosphorylated p53, which contains a nuclear export signal (NES). 14-3-3 dimer binds to phosphorylated E6 and p53 in anti-parallel manner, enabling 14-3-3 to keep both of these proteins in the cytoplasm.

this study is that it links p53 to the PBM function of E6. Several recent studies have shown that loss of the E6 PBM results in an inability of HPV genomes to remain as episomes (Lee & Laimins, 2004; Delury *et al*, 2013), but that this could be rescued in part by ablation of p53 (Lorenz *et al*, 2013; Brimer & Vande Pol, 2014). My results link these sets of observations, and suggest that the ability of E6 to interact with 14-3-3, and thereby inhibit p53 transcriptional transactivation, is a critical step in maintaining episomal genomes.

### **4.3 Overview of the involvement of E6 and E7 proteins in HPV-induced carcinogenesis**

The above studies have important implications on how E6 function is controlled both during the viral life cycle and during the development of malignancy. As the epithelial cells differentiate and move upwards from the basal cells, numerous changes take place in the cells. For survival, the virus has adopted capabilities to manipulate these events in order to allow cells to accomodate the virus, complete their life cycle, maintain their stability and avoid elimination from the host. In the early stages of viral infection, in the basal layers, the expression of HPV E6 and E7 results in the downregulation of the p53, pRB, PDZ-containing proteins and other pro-apoptotic proteins. This has been known for some time to favour the viral life cycle, enabling the virus to establish viral DNA replication and the eventual productive infection. As the cells differentiate, it was also shown that the suprabasal layer of infected epithelium is thicker than the non-infected epithelium. In

the cells harbouring HPV genomes, the majority of the cells in the suprabasal layers exist in an S-like or G2/M-like phases, where the PKA and AKT activity peaks. This means that in the suprabasal layer, the majority of HPV-16 and -18 E6 proteins are phosphorylated and stabilised, and they interact with 14-3-3. This interaction keeps 14-3-3 in the cytosol and, hence, potentially perturbs 14-3-3 nuclear-cytoplasmic shuttling ability. In the G2/M phase, HPV-18 E6 also degrades a significant portion of p53, whilst the remaining pool of p53 is kept in the cytoplasm by E6 through 14-3-3, preventing p53-mediated transcriptional transactivation. In addition, HPV-16 and -18 E6 target components of the Scribble polarity module (hDlg and Scribble) during S and G2/M phases in a slightly different manner. HPV-16 and -18 E6 target hDlg for degradation during G2/M, whilst HPV-18 E6 seems to target Scribble in G2/M, whilst HPV-16 E6 targets Scribble in both S and G2/M phases. In the long run, accumulation of chromosomal instability, lack of DNA repair mechanisms and improper cell cycle checkpoints induced by E6 and E7 may favour viral integration. However, it is important to note that viral DNA integration is not the ultimate goal of the virus. In cases where infection persists and the host immune response is suppressed, this can result in cervical carcinoma where the HPV E6 and E7 oncoproteins are highly expressed and the other viral proteins are lost. The phospho-status of E6 in this case is still unclear. However, it is tempting to speculate that changes in the E6 phosphorylation status, along with an increased population of cells in S-like and G2/M-like phases, might play an important role during the development of HPV-induced disease. All the above mentioned cellular events are expected to be enhanced, including

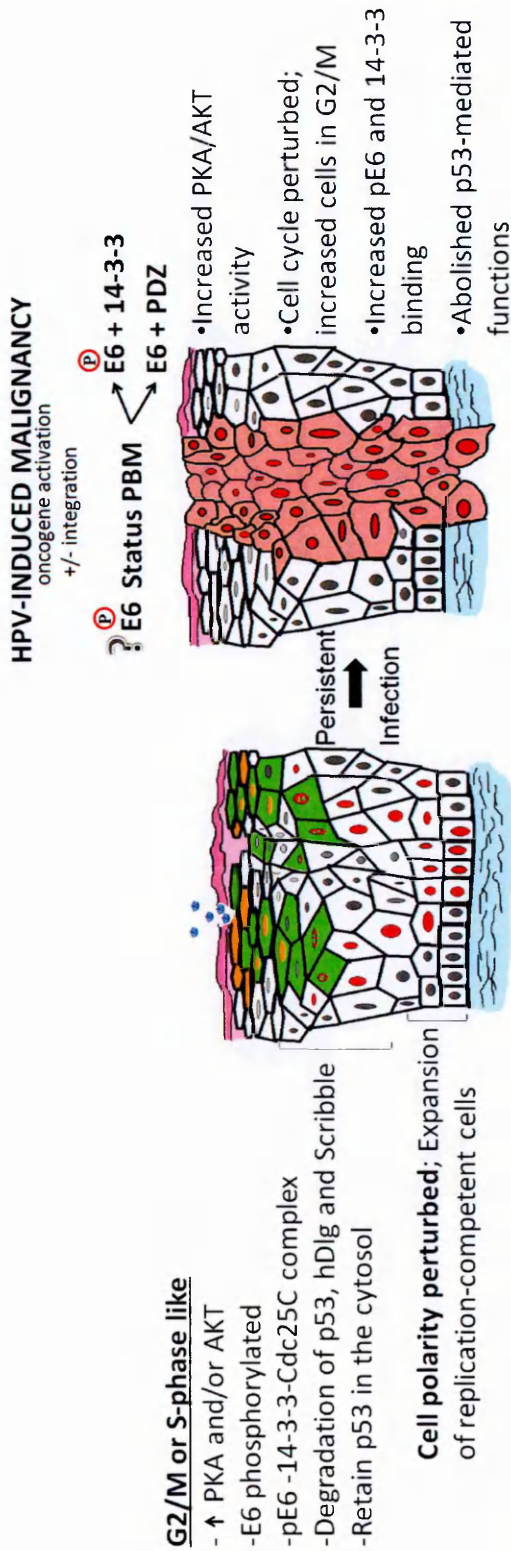
enhanced survival kinases which can phosphorylate E6, resulting in increased association with 14-3-3 and further retention of 14-3-3 in the cytosol, as well as increased degradation of hDlg, Scribble and p53. All these cellular changes in the infected epithelial cells, from low grade lesions to carcinoma are depicted in Figure 37. These events, in cooperation with E7, all together abrogate apoptosis and cell cycle checkpoints, inducing chromosomal abnormalities and instability, as well as increased survival signalling. Studies have shown that estrogen treatment can enhance HPV-induced carcinogenesis (Chung *et al*, 2008b; Chung & Lambert, 2009; Chung *et al*, 2013). Therefore, future studies should investigate whether or not estrogen has an effect on phospho-status of E6 in pre-cancerous lesions and carcinomas. This is of clinical relevance as it might be useful to predict the behaviour of the different types HPVs in cancer progression, and possibly in their life cycle.

#### 4.4 Future directions

These studies address the differential regulation of HR HPV E6s by PKA and AKT, which have an effect on E6 stability and its preferences in cellular protein targeting during different stages of the cell cycle. They also indicate that E6 phosphorylation has important implications for the viral life cycle and cause perturbation of p53 transcriptional transactivation mediated by 14-3-3 through E6-PBM. These results suggest future studies to investigate the following:

- i. To determine the ratio of phosphorylated HPV E6 to non-phosphorylated HPV E6. This can be done by performing stable isotope labelling by amino acids and cell culture (SILAC) or enzyme-linked immunosorbent assays (ELISA).
- ii. Identify in which epithelial layer HPV E6 is phosphorylated. This can be done by performing immunohistochemistry using organotypic culture, by comparing the phospho-status of wild type E6 and E6 R153A or  $\Delta$ PBM, mutants that are not phosphorylated by PKA or AKT. It would then be important to investigate if phospho-E6 colocalises with 14-3-3 and p53 or 14-3-3 and Cdc25 complex in the cytosol.
- iii. Since the ubiquitously expressed 14-3-3 proteins are involved in multiple cellular events, it would also be interesting to identify other 14-3-3 functions that can be perturbed by E6.
- iv. Identify other kinases that could potentially phosphorylate HR HPV E6s besides PKA and AKT.
- v. As HPV E6 is involved in manipulating signalling pathways and estrogen has been reported to be one of the co-factors that enhances HPV-induced carcinogenesis, it would be very interesting to study whether E6 is involved directly in targeting signalling components and whether estrogen has an effect on the phospho-status of HPV E6.

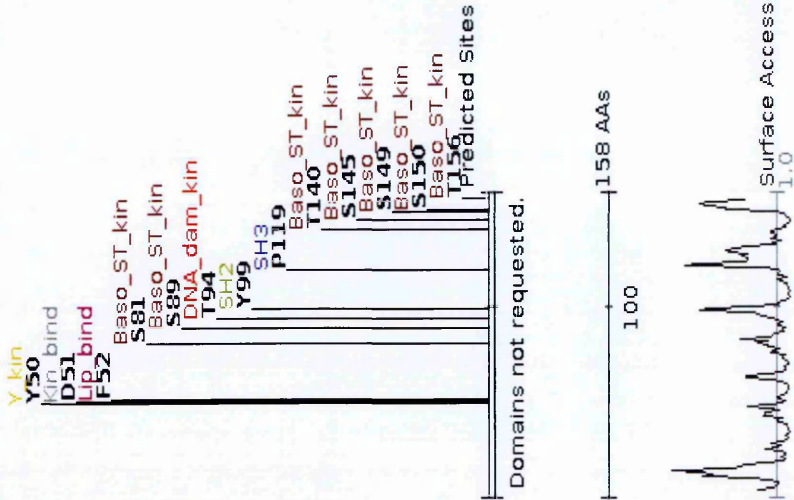




**Figure 37. Overview of the role of HPV E6 in initial infection and in cervical carcinomas.** Upon HPV infection, the expression of HPV E6 (red) perturbs cell polarity by degrading PDZ-containing proteins through E6-PBM, and hence allowing cells to differentiate to suprabasal layers. In suprabasal layers, the cells exist in an S-phase-like or G2/M-like phase. The levels of survival kinases, such as PKA and AKT increase, resulting in increased E6 phosphorylation and stability. This increases 14-3-3 recognition. Phospho-E6 retains 14-3-3-Cdc25C and 14-3-3-p53 complexes in the cytosol, and it also degrades p53, hDlg and Scribble. This abrogates apoptosis and cell cycle checkpoints, and increases survival signalling. All these events are important to create a conducive environment for the virus to continue viral genome amplification (green represents early genes and orange represents late genes) and viral synthesis (blue). Upon persistent infection, lack of host immune elimination and accumulation of genetic errors, the lesions can progress to carcinomas, with the HPV E6 and E7 oncoproteins overexpressed. However, the phosphorylation status of E6 is unknown. It would be expected that in the increased population of cells in S-like and G2/M-like phases, having increased PKA/AKT activity, the cell cycle and cell polarity will be perturbed, in addition to loss of tumour suppressors and increased 14-3-3 association. E6-14-3-3 interaction is crucial for E6 stability and establishes a relationship where E6 can augment 14-3-3's role in carcinogenesis where its normal function in the cells is perturbed by E6.

APPENDIX (Scansite prediction results for HPV E6 proteins)

HPV-16 E6 (Alpha 7)



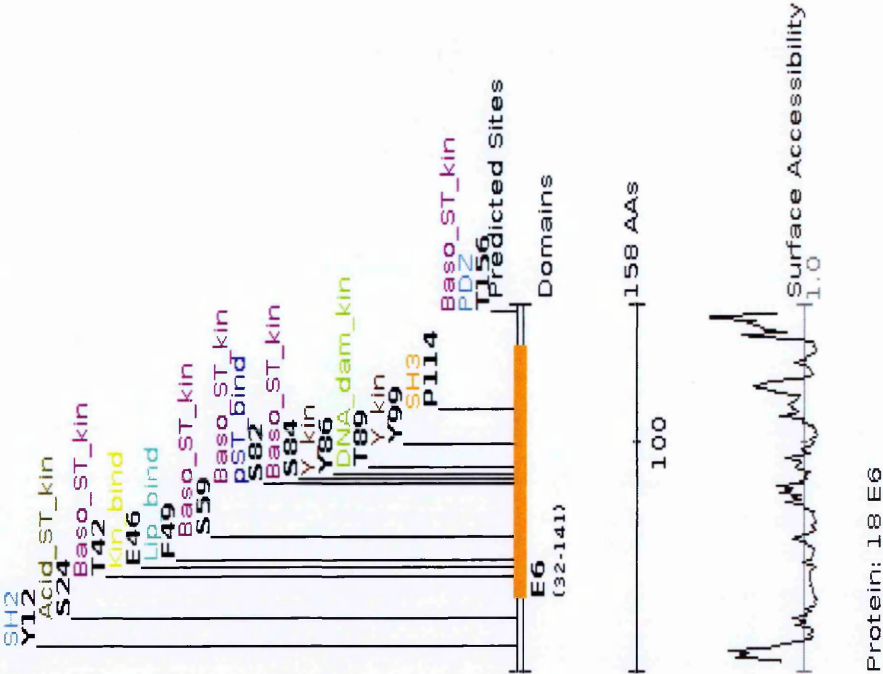
Score	Percentile	Motif	Motif group	Site	Sequence	Surface Accessibility	Gene Info
<u>0.711</u>	4.551%	Akt Kinase (Akt_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	S89	<u>EYRHYCYsLYGTT</u> <u>LE</u>	0.3299	<u>AKT1</u>
<u>0.675</u>	3.133%	Akt Kinase (Akt_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	S145	<u>RWTGRCMsCCRSS</u> <u>RT</u>	0.0917	<u>AKT1</u>
<u>0.527</u>	0.490%	Akt Kinase (Akt_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	T156	<u>SSRTRREtQL*****</u>	3.3033	<u>AKT1</u>
<u>0.613</u>	1.976%	Aurora A (AuroA)	Basophilic serine/threonine kinase (Baso_ST_kin)	S89	<u>EYRHYCYsLYGTT</u> <u>LE</u>	0.3299	<u>AURKA</u>
<u>0.626</u>	2.260%	Aurora A (AuroA)	Basophilic serine/threonine kinase (Baso_ST_kin)	S145	<u>RWTGRCMsCCRSS</u> <u>RT</u>	0.0917	<u>AURKA</u>
<u>0.680</u>	3.850%	Aurora A (AuroA)	Basophilic serine/threonine kinase (Baso_ST_kin)	S150	<u>CMSCCRSSsRTRRET</u> <u>Q</u>	4.4628	<u>AURKA</u>
<u>0.616</u>	1.450%	Aurora B (AuroB)	Basophilic	T140	<u>HNIRGRWtGRCMS</u>	0.7077	<u>AURKB</u>

<u>0.654</u>	2.189%	Aurora B (AuroB)	serine/threonine kinase (Baso_ST_kin) Basophilic serine/threonine kinase (Baso_ST_kin)	S150	<u>CMSCCRsSRTTRRET</u> Q	4.4628	<u>AURKB</u>
<u>0.699</u>	1.001%	Clk2 Kinase (Clk2_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	S145	<u>RWTGRCMsCCRSS</u> RT	0.0917	<u>CLK2</u>
<u>0.776</u>	2.337%	Clk2 Kinase (Clk2_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	S149	<u>RCMSCCRsSRTTRRE</u> T	1.2214	<u>CLK2</u>
<u>0.844</u>	4.934%	Clk2 Kinase (Clk2_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	S150	<u>CMSCCRsSRTTRRET</u> Q	4.4628	<u>CLK2</u>
<u>0.526</u>	3.943%	PKC alpha/beta/gamma (PKC_common)	Basophilic serine/threonine kinase (Baso_ST_kin)	S145	<u>RWTGRCMsCCRSS</u> RT	0.0917	<u>PRKCA</u>
<u>0.521</u>	3.624%	PKC alpha/beta/gamma (PKC_common)	Basophilic serine/threonine kinase (Baso_ST_kin)	S149	<u>RCMSCCRsSRTTRRE</u> T	1.2214	<u>PRKCA</u>
<u>0.522</u>	2.042%	PKC zeta (PKC_zeta)	Basophilic	S81	<u>LKFYSKIsEYRHYC</u>	2.2882	<u>PRKCZ</u>



<u>0.497</u>	1.281%	Protein Kinase A (PKA_Kin)	serine/threonine kinase (Baso_ST_kin)	<u>Y</u>		
<u>0.620</u>	4.361%	DNA PK (DNA_PK)	Basophilic serine/threonine kinase (Baso_ST_kin)	T156	<u>SSRTRREtQL*****</u>	3.3033 <u>PRKACG</u>
<u>0.718</u>	1.452%	PDK1 Binding (PDK1_Bind)	DNA damage kinase group (DNA_dam_kin)	T94	<u>CYSLYGTtLEQQY</u> <u>NK</u>	1.1683 <u>PRKDC</u>
<u>0.790</u>	2.677%	PIP3-binding PH (PIP3_PH)	Kinase binding site group (Kin_bind)	D51	<u>LLRREVYdFAFRDL</u> <u>C</u>	0.3371 <u>PDPK1</u>
<u>0.540</u>	3.156%	Crk SH2 (Crk_SH2)	Lipid binding group (Lip_bind)	F52	<u>LRREVYDfAFRDL</u> <u>CI</u>	0.8897 <u>PIP3-E</u>
<u>0.713</u>	3.938%	p85 SH3 mode2 (p85_SH3_m2)	Src homology 2 group (SH2)	Y99	<u>GTTLEQQyNKPLC</u> <u>DL</u>	5.5616 <u>CRK</u>
<u>0.570</u>	3.694%	Lck Kinase (Lck_Kin)	Src homology 3 group (SH3)	P119	<u>NCQKPLCpEEKQR</u> <u>HL</u>	0.9396 <u>PIK3R</u>
			Tyrosine kinase group (Y_kin)	Y50	<u>QLLRREVyDFAFR</u> <u>DL</u>	0.6743 <u>LCK</u>

HPV-18 E6 (Alpha 9)



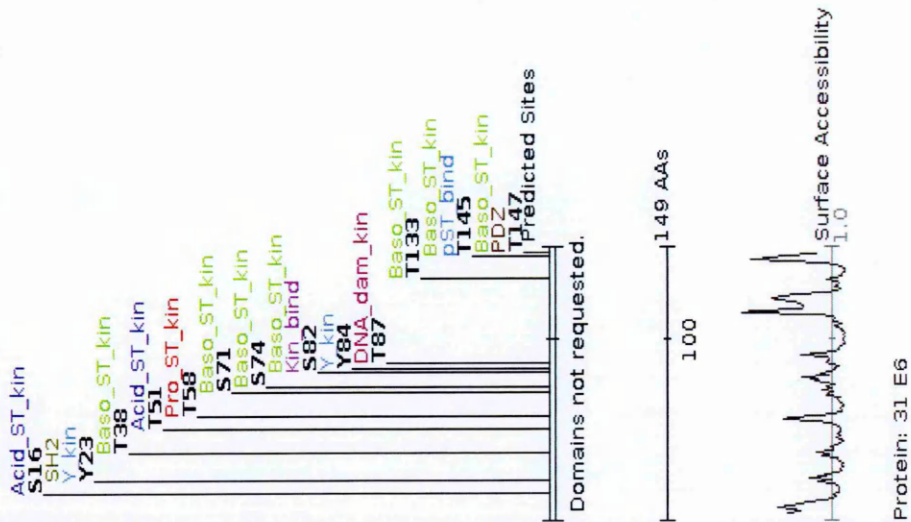
Score	Percentile	Motif	Motif group	Site	Sequence	Surface Accessibility	Gene Info
<u>0.342</u>	0.963%	PLK1 Kinase (PLK1)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	S24	<u>LCTELNTsLQDIEIT</u>	1.7654	<u>PLK1</u>
<u>0.717</u>	4.844%	Akt Kinase (Akt_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	S84	<u>ELRHYSDsVYGDITLE</u>	0.7910	<u>AKT1</u>
<u>0.569</u>	1.209%	Aurora A (AuroA)	Basophilic serine/threonine kinase (Baso_ST_kin)	S59	<u>LFVVYRDsIPHAACH</u>	1.4816	<u>AURKA</u>
<u>0.616</u>	1.446%	Aurora B (AuroB)	Basophilic serine/threonine kinase (Baso_ST_kin)	S59	<u>LFVVYRDsIPHAACH</u>	1.4816	<u>AURKB</u>
<u>0.568</u>	3.218%	Calmodulin dependent Kinase 2 (Cam_Kin2)	Basophilic serine/threonine kinase (Baso_ST_kin)	S82	<u>IRELRHYsDSVYGDT</u>	1.0876	<u>CAMK2G</u>

Score	Percentile	Motif	Motif group	Site	Sequence	Surface Accessibility	Gene Info
<u>0.754</u>	1.832%	Clk2 Kinase (Clk2_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	S82	<u>IRELRHYsDSVYGDT</u>	1.0876	<u>CLK2</u>
<u>0.465</u>	1.478%	PKC mu (PKC_mu)	Basophilic serine/threonine kinase (Baso_ST_kin)	T42	<u>CKTVLELEVFEEFAF</u>	0.5258	<u>PRKCM</u>
<u>0.463</u>	0.912%	Protein Kinase A (PKA_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	T156	<u>RLQRRREIQV*****</u>	2.9730	<u>PRKAC</u> <u>G</u>
<u>0.579</u>	2.719%	ATM Kinase (ATM_Kin)	DNA damage kinase group (DNA_dam_kin)	T89	<u>SDSVYGDILLEKLTNT</u>	1.5612	<u>ATM</u>
<u>0.569</u>	2.119%	DNA PK (DNA_PK)	DNA damage kinase group (DNA_dam_kin)	T89	<u>SDSVYGDILLEKLTNT</u>	1.5612	<u>PRKDC</u>
<u>0.670</u>	1.355%	PKD1 Binding (PKD1_Bind)	Kinase binding site group (Kin_bind)	E46	<u>LELTEVFEEFAFKDLF</u>	0.1932	<u>PDPK1</u>
<u>0.809</u>	3.488%	PIP3-binding PH (pip3_PH)	Lipid binding group (Lip_bind)	F49	<u>TEVFEEFAFKDLFVVY</u>	0.4781	<u>PIP3-E</u>
<u>0.339</u>	2.347%	PDZ class 1 (PDZ_class1)	PDZ domain binding group (PDZ)	T156	<u>RLQRRREIQV*****</u>	2.9730	<u>PDZK1</u>
<u>0.536</u>	2.241%	14-3-3 Mode 1 (1433_m1)	Phosphoserine/threonine binding group	S82	<u>IRELRHYsDSVYGDT</u>	1.0876	<u>YWHAZ</u>



Score	Percentile	Motif	Motif group	Site	Sequence	Surface Accessibility	Gene Info
<u>0.522</u>	2.587%	Crk SH2 (Crk_SH2)	(pST_bind) Src homology 2 group (SH2)	Y12	<u>EDPTRRP</u> <u>yKLPDLCT</u>	2.7733	<u>CRK</u>
<u>0.637</u>	1.434%	p85 SH3 mode2 (p85_SH3_m2)	Src homology 3 group (SH3)	P114	<u>RCQKPLN</u> <u>pAEKLRHL</u>	1.7075	<u>PIK3R1</u>
<u>0.508</u>	1.353%	Lck Kinase (Lck_Kin)	Tyrosine kinase group (Y_kin)	Y86	<u>RHYSDSV</u> <u>yGDTLEKL</u>	0.8519	<u>LCK</u>
<u>0.568</u>	3.581%	Lck Kinase (Lck_Kin)	Tyrosine kinase group (Y_kin)	Y99	<u>KLTNTGL</u> <u>yNLLIRCL</u>	0.3328	<u>LCK</u>
<u>0.547</u>	1.841%	Src Kinase (Src_Kin)	Tyrosine kinase group (Y_kin)	Y86	<u>RHYSDSV</u> <u>yGDTLEKL</u>	0.8519	<u>SRC</u>

HPV-31 E6 (Alpha-9)



Score	Percentile	Motif	Motifgroup	Site	Sequence	Surface Accessibility	Gene Info
<u>0.500</u>	4.587%	Casein Kinase 1 (Casn_Kin1)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	T51	<u>DFAFTDLtIV</u> <u>YRDDT</u>	0.3713	<u>CSNK1G2</u>
<u>0.587</u>	14.554%	Casein Kinase 1 (Casn_Kin1)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	T86	<u>YRYSVYGtT</u> <u>LEKLTN</u>	1.0571	<u>CSNK1G2</u>
<u>0.630</u>	8.772%	Casein Kinase 2 (Casn_Kin2)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	S16	<u>PRKLHELsS</u> <u>ALEIPY</u>	0.4897	<u>CSNK2B</u>
<u>0.518</u>	4.906%	PLK1 Kinase (PLK1)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	S16	<u>PRKLHELsS</u> <u>ALEIPY</u>	0.4897	<u>PLK1</u>
<u>0.299</u>	0.430%	PLK1 Kinase (PLK1)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	T51	<u>DFAFTDLtIV</u> <u>YRDDT</u>	0.3713	<u>PLK1</u>
<u>0.826</u>	13.760%	AMP_Kinase (AMPK)	Basophilic serine/threonine kinase (Baso_ST_kin)	S17	<u>RKLHELsSA</u> <u>LEIPYD</u>	0.4897	<u>PRKAA1</u>
<u>0.602</u>	0.681%	AMP_Kinase (AMPK)	Basophilic serine/threonine kinase (Baso_ST_kin)	T145	<u>ACWRRRPrtE</u> <u>TOV***</u>	4.3356	<u>PRKAA1</u>
<u>0.756</u>	7.242%	Akt Kinase (Akt_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	T58	<u>TIVYRDDtP</u> <u>HGVCTK</u>	1.9206	<u>AKT1</u>
<u>0.765</u>	7.997%	Akt Kinase (Akt_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	S71	<u>TKCLRFYsK</u> <u>VSEFRW</u>	0.8289	<u>AKT1</u>

<u>0.677</u>	3.187%	Akt Kinase (Akt_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	S82	<u>EFRWYRYsV</u> <u>YGTTL</u>	1.0847	<u>AKT1</u>
<u>0.761</u>	7.640%	Akt Kinase (Akt_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	T105	<u>DLLJRCItCO</u> <u>RPLCP</u>	0.2260	<u>AKT1</u>
<u>0.557</u>	0.745%	Akt Kinase (Akt_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	T147	<u>WRRPRTEtQ</u> <u>V****</u>	2.1906	<u>AKT1</u>
<u>0.790</u>	10.737%	Aurora A (AuroA)	Basophilic serine/threonine kinase (Baso_ST_kin)	T51	<u>DFAFTDLtIV</u> <u>YRDDT</u>	0.3713	<u>AURKA</u>
<u>0.416</u>	0.167%	Aurora A (AuroA)	Basophilic serine/threonine kinase (Baso_ST_kin)	S82	<u>EFRWYRYsV</u> <u>YGTTL</u>	1.0847	<u>AURKA</u>
<u>0.807</u>	12.363%	Aurora A (AuroA)	Basophilic serine/threonine kinase (Baso_ST_kin)	T105	<u>DLLJRCItCO</u> <u>RPLCP</u>	0.2260	<u>AURKA</u>
<u>0.818</u>	13.610%	Aurora A (AuroA)	Basophilic serine/threonine kinase (Baso_ST_kin)	T133	<u>HNIGGRWtG</u> <u>RCIACW</u>	0.7077	<u>AURKA</u>
<u>0.845</u>	10.082%	Aurora B (AuroB)	Basophilic serine/threonine kinase (Baso_ST_kin)	T51	<u>DFAFTDLtIV</u> <u>YRDDT</u>	0.3713	<u>AURKB</u>
<u>0.636</u>	1.795%	Aurora B (AuroB)	Basophilic serine/threonine kinase (Baso_ST_kin)	S82	<u>EFRWYRYsV</u> <u>YGTTL</u>	1.0847	<u>AURKB</u>
<u>0.825</u>	8.649%	Aurora B (AuroB)	Basophilic serine/threonine kinase (Baso_ST_kin)	T92	<u>GTTLEKLtN</u> <u>KGICDL</u>	1.8028	<u>AURKB</u>
<u>0.780</u>	6.200%	Aurora B (AuroB)	Basophilic serine/threonine kinase (Baso_ST_kin)	T105	<u>DLLJRCItCO</u> <u>RPLCP</u>	0.2260	<u>AURKB</u>
<u>0.627</u>	1.622%	Aurora B (AuroB)	Basophilic serine/threonine kinase (Baso_ST_kin)	T133	<u>HNIGGRWtG</u> <u>RCIACW</u>	0.7077	<u>AURKB</u>
<u>0.566</u>	3.090%	Calmodulin dependent Kinase 2 (Cam_Kin2)	Basophilic serine/threonine kinase (Baso_ST_kin)	T38	<u>VYCKGOLtE</u> <u>TEVLDF</u>	2.0446	<u>CAMK2G</u>



<u>0.938</u>	12.904%	Clk2 Kinase (Clk2_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	S16	<u>PRKLHELsS</u> <u>ALEIPY</u>	0.4897	<u>CLK2</u>
<u>0.755</u>	1.861%	Clk2 Kinase (Clk2_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	S71	<u>TKCLRFYsK</u> <u>VSEFRW</u>	0.8289	<u>CLK2</u>
<u>0.557</u>	6.657%	PKC alpha/beta/gamma (PKC_common)	Basophilic serine/threonine kinase (Baso_ST_kin)	T87	<u>RYSVYGTtL</u> <u>EKLtNK</u>	1.3492	<u>PRKCA</u>
<u>0.585</u>	14.722%	PKC epsilon (PKC_epsilon)	Basophilic serine/threonine kinase (Baso_ST_kin)	S16	<u>PRKLHELsS</u> <u>ALEIPY</u>	0.4897	<u>PRKCE</u>
<u>0.562</u>	10.442%	PKC epsilon (PKC_epsilon)	Basophilic serine/threonine kinase (Baso_ST_kin)	T51	<u>DFAFTDLtIV</u> <u>YRDDT</u>	0.3713	<u>PRKCE</u>
<u>0.562</u>	10.442%	PKC epsilon (PKC_epsilon)	Basophilic serine/threonine kinase (Baso_ST_kin)	S82	<u>EFRWYRYsV</u> <u>YGTtLE</u>	1.0847	<u>PRKCE</u>
<u>0.582</u>	11.271%	PKC mu (PKC_mu)	Basophilic serine/threonine kinase (Baso_ST_kin)	S16	<u>PRKLHELsS</u> <u>ALEIPY</u>	0.4897	<u>PRKCM</u>
<u>0.596</u>	14.030%	PKC mu (PKC_mu)	Basophilic serine/threonine kinase (Baso_ST_kin)	S17	<u>RKLHELsSA</u> <u>LEIPYD</u>	0.4897	<u>PRKCM</u>
<u>0.585</u>	6.460%	PKC zeta (PKC_zeta)	Basophilic serine/threonine kinase (Baso_ST_kin)	S17	<u>RKLHELsSA</u> <u>LEIPYD</u>	0.4897	<u>PRKCZ</u>
<u>0.453</u>	0.512%	PKC zeta (PKC_zeta)	Basophilic serine/threonine kinase (Baso_ST_kin)	S74	<u>LRFYsKVsE</u> <u>FRWYRY</u>	1.3389	<u>PRKCZ</u>
<u>0.598</u>	7.699%	PKC zeta (PKC_zeta)	Basophilic serine/threonine kinase (Baso_ST_kin)	T86	<u>YRYSVYGtT</u> <u>LEKLtN</u>	1.0571	<u>PRKCZ</u>
<u>0.707</u>	12.035%	Protein Kinase A (PKA_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	T58	<u>TIVYRDDtP</u> <u>HGVCTK</u>	1.9206	<u>PRKACG</u>
<u>0.664</u>	8.386%	Protein Kinase A (PKA_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	S71	<u>TKCLRFYsK</u> <u>VSEFRW</u>	0.8289	<u>PRKACG</u>

<u>0.626</u>	5.751%	Protein Kinase A (PKA_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	S82	<u>EFRWRYsV</u> <u>YGTTLE</u>	1.0847	<u>PRKACG</u>
<u>0.728</u>	13.818%	Protein Kinase A (PKA_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	T133	<u>HNIGGRWtG</u> <u>RCIACW</u>	0.7077	<u>PRKACG</u>
<u>0.695</u>	13.792%	ATM Kinase (ATM_Kin)	DNA damage kinase group (DNA_dam_kin)	T38	<u>VYCKGOLtE</u> <u>TEVLDF</u>	2.0446	<u>ATM</u>
<u>0.667</u>	9.672%	ATM Kinase (ATM_Kin)	DNA damage kinase group (DNA_dam_kin)	T48	<u>EVLDFAFtD</u> <u>LTIVYR</u>	0.5750	<u>ATM</u>
<u>0.663</u>	9.201%	ATM Kinase (ATM_Kin)	DNA damage kinase group (DNA_dam_kin)	T51	<u>DFAFTDLtIV</u> <u>YRDDT</u>	0.3713	<u>ATM</u>
<u>0.604</u>	4.023%	ATM Kinase (ATM_Kin)	DNA damage kinase group (DNA_dam_kin)	T87	<u>RYSVYGTtL</u> <u>EKLtNK</u>	1.3492	<u>ATM</u>
<u>0.707</u>	12.504%	DNA PK (DNA_PK)	DNA damage kinase group (DNA_dam_kin)	T38	<u>VYCKGOLtE</u> <u>TEVLDF</u>	2.0446	<u>PRKDC</u>
<u>0.609</u>	3.790%	DNA PK (DNA_PK)	DNA damage kinase group (DNA_dam_kin)	T87	<u>RYSVYGTtL</u> <u>EKLtNK</u>	1.3492	<u>PRKDC</u>
<u>0.692</u>	10.535%	DNA PK (DNA_PK)	DNA damage kinase group (DNA_dam_kin)	T147	<u>WRRPRTEtQ</u> <u>V*****</u>	2.1906	<u>PRKDC</u>
<u>0.847</u>	11.819%	Erk D-domain (ErkDD)	Kinase binding site group (Kin_bind)	L15	<u>RPRKLHEISS</u> <u>ALtEP</u>	0.8080	<u>MAPK1</u>
<u>0.797</u>	7.572%	Erk D-domain (ErkDD)	Kinase binding site group (Kin_bind)	I128	<u>KKKRFHNIg</u> <u>GRWtGR</u>	0.7002	<u>MAPK1</u>
<u>1.018</u>	13.008%	PDK1 Binding (PDK1_Bind)	Kinase binding site group (Kin_bind)	T51	<u>DFAFTDLtIV</u> <u>YRDDT</u>	0.3713	<u>PDPK1</u>
<u>0.795</u>	1.776%	PDK1 Binding (PDK1_Bind)	Kinase binding site group (Kin_bind)	S82	<u>EFRWRYsV</u> <u>YGTTLE</u>	1.0847	<u>PDPK1</u>

<u>0.912</u>	11.384%	PIP3-binding PH (PIP3_PH)	Lipid binding group (Lip_bind)	F76	<u>FYSKVSEfR</u> <u>WYRYSV</u>	1.4861	<u>PIP3-E</u>
<u>0.865</u>	6.762%	PIP3-binding PH (PIP3_PH)	Lipid binding group (Lip_bind)	W132	<u>FHNIGGRwT</u> <u>GRCIAC</u>	1.3065	<u>PIP3-E</u>
<u>0.614</u>	1.335%	PDZ (nNOS) class 1 (PDZ_nNOS_1)	PDZ domain binding group (PDZ)	T147	<u>WRRPRTEtQ</u> <u>V****</u>	2.1906	<u>NOS1</u>
<u>0.339</u>	2.347%	PDZ class 1 (PDZ_class1)	PDZ domain binding group (PDZ)	T147	<u>WRRPRTEtQ</u> <u>V****</u>	2.1906	<u>PDZK1</u>
<u>0.753</u>	14.691%	14-3-3 Mode 1 (1433_m1)	Phosphoserine/threonine binding group (pST_bind)	T58	<u>TIVYRDDtP</u> <u>HGVCTK</u>	1.9206	<u>YW1IAZ</u>
<u>0.711</u>	10.686%	14-3-3 Mode 1 (1433_m1)	Phosphoserine/threonine binding group (pST_bind)	S71	<u>TKCLRFYsK</u> <u>VSEFRW</u>	0.8289	<u>YWHAZ</u>
<u>0.705</u>	10.192%	14-3-3 Mode 1 (1433_m1)	Phosphoserine/threonine binding group (pST_bind)	T105	<u>DLLIRCIcQ</u> <u>RPLCP</u>	0.2260	<u>YWHAZ</u>
<u>0.507</u>	1.691%	14-3-3 Mode 1 (1433_m1)	Phosphoserine/threonine binding group (pST_bind)	T145	<u>ACWRRRPtE</u> <u>TOV***</u>	4.3356	<u>YWHAZ</u>
<u>0.716</u>	14.479%	Shc PTB (Shc_PT B)	Phosphotyrosine binding group (PTB)	Y32	<u>ELRLNCVvC</u> <u>KGQLTE</u>	0.1516	<u>SHC1</u>
<u>0.668</u>	4.737%	CDK1 motif 1 - [ST]Px[KR]x (CDK1_1)	Proline-dependent serine/threonine kinase group (Pro_ST_kin)	T58	<u>TIVYRDDtP</u> <u>HGVCTK</u>	1.9206	<u>CDK1</u>
<u>1.003</u>	12.049%	CDK1 motif 1 - [ST]Px[KR]x (CDK1_1)	Proline-dependent serine/threonine kinase group (Pro_ST_kin)	T87	<u>RYSVYGTtL</u> <u>EKLTK</u>	1.3492	<u>CDK1</u>
<u>0.931</u>	8.485%	CDK1 motif 1 - [ST]Px[KR]x	Proline-dependent serine/threonine kinase	T105	<u>DLLIRCIcQ</u> <u>RPLCP</u>	0.2260	<u>CDK1</u>

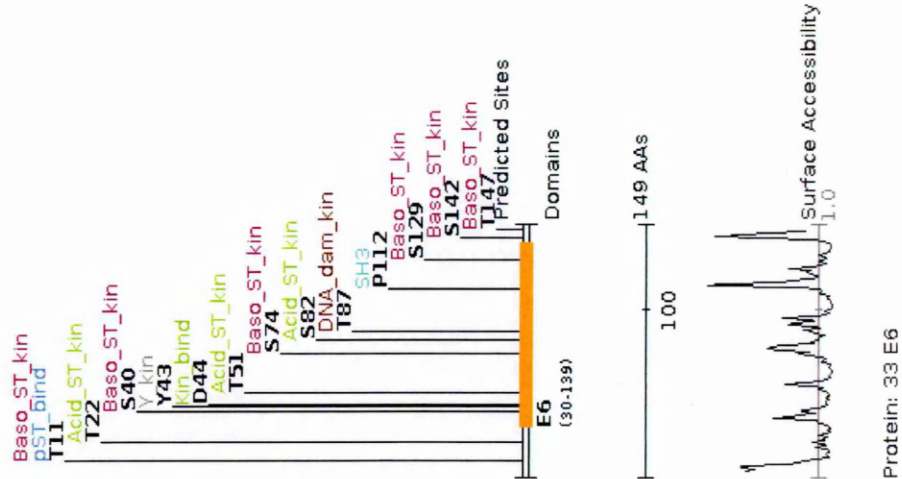
<u>0.649</u>	4.740%	(CDK1_1) CDK1 motif 2 - [ST]pxxK (CDK1_2)	group (Pro_ST_kin) Proline-dependent serine/threonine kinase group (Pro_ST_kin)	T58	<u>TIVYRDDtP</u> <u>HGVCTK</u>	1.9206	<u>CDK1</u>
<u>1.116</u>	14.617%	CDK1 motif 2 - [ST]pxxK (CDK1_2)	Proline-dependent serine/threonine kinase group (Pro_ST_kin)	T105	<u>DLLIRCItCQ</u> <u>RPLCP</u>	0.2260	<u>CDK1</u>
<u>0.663</u>	4.725%	Cdc2 Kinase (Cdc2_Kin)	Proline-dependent serine/threonine kinase group (Pro_ST_kin)	T58	<u>TIVYRDDtP</u> <u>HGVCTK</u>	1.9206	<u>CDC2</u>
<u>0.629</u>	4.587%	Cdk5 Kinase (Cdk5_Kin)	Proline-dependent serine/threonine kinase group (Pro_ST_kin)	T58	<u>TIVYRDDtP</u> <u>HGVCTK</u>	1.9206	<u>CDK5</u>
<u>0.755</u>	12.837%	Erk1 Kinase (Erk1_Kin)	Proline-dependent serine/threonine kinase group (Pro_ST_kin)	S17	<u>RKLHELSSA</u> <u>LEIPYD</u>	0.4897	<u>MAPK3</u>
<u>0.764</u>	13.910%	Erk1 Kinase (Erk1_Kin)	Proline-dependent serine/threonine kinase group (Pro_ST_kin)	T38	<u>VYCKGQLtE</u> <u>TEVLDF</u>	2.0446	<u>MAPK3</u>
<u>0.752</u>	12.423%	Erk1 Kinase (Erk1_Kin)	Proline-dependent serine/threonine kinase group (Pro_ST_kin)	T58	<u>TIVYRDDtP</u> <u>HGVCTK</u>	1.9206	<u>MAPK3</u>
<u>0.643</u>	8.515%	Abl SH2 (Abl_SH2)	Src homology 2 group (SH2)	Y23	<u>SSALEIPvDE</u> <u>LRLNC</u>	0.9283	<u>ABL1</u>
<u>0.590</u>	8.398%	Fgr SH2 (Fgr_SH2)	Src homology 2 group (SH2)	Y23	<u>SSALEIPvDE</u> <u>LRLNC</u>	0.9283	<u>FGR</u>
<u>0.611</u>	10.677%	Fyn SH2 (Fyn_SH2)	Src homology 2 group	Y23	<u>SSALEIPvDE</u>	0.9283	<u>FYN</u>



<u>0.580</u>	12.918%	Lck SH2 (Lck_SH2)	(SH2)	Src homology 2 group (SH2)	Y23	<u>SSALEIPvDE</u> <u>LRLNC</u>	0.9283	<u>LCK</u>
<u>0.510</u>	1.992%	Nck SH2 (Nck_SH2)		Src homology 2 group (SH2)	Y23	<u>SSALEIPvDE</u> <u>LRLNC</u>	0.9283	<u>NCK1</u>
<u>0.532</u>	9.244%	PLCg N-terminal SH2 (PLCg_NSH2)		Src homology 2 group (SH2)	Y23	<u>SSALEIPvDE</u> <u>LRLNC</u>	0.9283	<u>PLCG1</u>
<u>0.460</u>	2.106%	SHIP SH2 (SHIP_SH2)		Src homology 2 group (SH2)	Y23	<u>SSALEIPvDE</u> <u>LRLNC</u>	0.9283	<u>INPP5D</u>
<u>0.541</u>	7.822%	SHIP SH2 (SHIP_SH2)		Src homology 2 group (SH2)	Y54	<u>FTDLTIVvR</u> <u>DDTPHG</u>	1.0205	<u>INPP5D</u>
<u>0.586</u>	7.535%	Shc SH2 (Shc_SH2)		Src homology 2 group (SH2)	Y23	<u>SSALEIPvDE</u> <u>LRLNC</u>	0.9283	<u>SHC1</u>
<u>0.484</u>	3.051%	Src SH2 (Src_SH2)		Src homology 2 group (SH2)	Y23	<u>SSALEIPvDE</u> <u>LRLNC</u>	0.9283	<u>SRC</u>
<u>0.748</u>	13.670%	Amphiphysin SH3 (Amphi_SH3)		Src homology 3 group (SH3)	P109	<u>RCITCQRpL</u> <u>CPEEKQ</u>	0.8216	<u>AMPH</u>
<u>0.713</u>	8.619%	Amphiphysin SH3 (Amphi_SH3)		Src homology 3 group (SH3)	P112	<u>TCORPLCpE</u> <u>EKORHL</u>	0.9396	<u>AMPH</u>
<u>0.683</u>	7.319%	PLCg SH3 (PLCg_SH3)		Src homology 3 group (SH3)	p9	<u>FKNPAERpR</u> <u>KLHELs</u>	3.8827	<u>PLCG1</u>
<u>0.754</u>	7.822%	p85 SH3 model (p85_SH3_m1)		Src homology 3 group (SH3)	P109	<u>RCITCQRpL</u> <u>CPEEKQ</u>	0.8216	<u>PIK3R1</u>
<u>0.742</u>	5.586%	p85 SH3 model2		Src homology 3 group	P112	<u>TCORPLCpE</u>	0.9396	<u>PIK3R1</u>

	(p85_SH3_m2)	(SH3)				
<u>0.606</u>	4.614%	Abl Kinase (Abl_Kin)	Tyrosine kinase group (Y_kin)	Y84	<u>EKQRHL</u> <u>RWYRYSVY</u> <u>GTTLEKL</u>	0.7362 <u>ABL1</u>
<u>0.621</u>	7.543%	Lck Kinase (Lck_Kin)	Tyrosine kinase group (Y_kin)	Y23	<u>SSALEIPyDE</u> <u>LRLNC</u>	0.9283 <u>LCK</u>
<u>0.578</u>	4.119%	Lck Kinase (Lck_Kin)	Tyrosine kinase group (Y_kin)	Y84	<u>RWYRYSVY</u> <u>GTTLEKL</u>	0.7362 <u>LCK</u>
<u>0.576</u>	2.865%	Src Kinase (Src_Kin)	Tyrosine kinase group (Y_kin)	Y23	<u>SSALEIPyDE</u> <u>LRLNC</u>	0.9283 <u>SRC</u>
<u>0.677</u>	11.343%	Src Kinase (Src_Kin)	Tyrosine kinase group (Y_kin)	Y84	<u>RWYRYSVY</u> <u>GTTLEKL</u>	0.7362 <u>SRC</u>

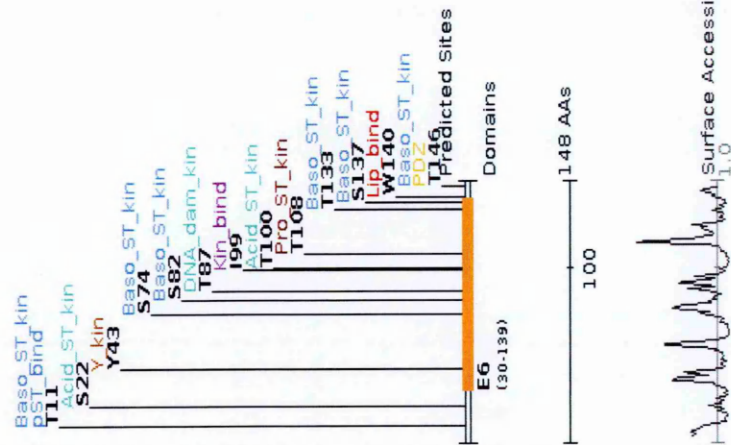
HPV-33 E6 (Alpha-9)



Score	Percentile	Motif	Motifgroup	Site	Sequence	Surface Accessibility	Gene Info
<u>0.268</u>	0.220%	PLK1 Kinase (PLK1)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	T22	<u>L</u> COALET <u>u</u> HNI <u>EL</u> Q	1.3167	<u>PLK1</u>
<u>0.418</u>	2.442%	PLK1 Kinase (PLK1)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	T51	<u>D</u> FAFADL <u>t</u> VVY <u>RE</u> GN	0.3932	<u>PLK1</u>
<u>0.487</u>	4.074%	PLK1 Kinase (PLK1)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	S82	<u>E</u> YRHYN <u>s</u> VYGN T <u>LE</u>	0.9249	<u>PLK1</u>
<u>0.727</u>	1.372%	Clk2 Kinase (Clk2_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	S142	<u>R</u> CAACW <u>R</u> sRR <u>RE</u> T <u>AL</u>	4.7522	<u>CLK2</u>
<u>0.506</u>	2.753%	PKC alpha/beta/gamma (PKC_common)	Basophilic serine/threonine kinase (Baso_ST_kin)	S129	<u>N</u> KRFHNI <u>s</u> GR <u>WA</u> G <u>RC</u>	0.7327	<u>PRKCA</u>
<u>0.560</u>	4.381%	PKC zeta (PKC_zeta)	Basophilic serine/threonine kinase (Baso_ST_kin)	S40	<u>C</u> KKPLOR <u>s</u> EVY <u>DF</u> A <u>F</u>	2.0981	<u>PRKCZ</u>
<u>0.560</u>	4.381%	PKC zeta (PKC_zeta)	Basophilic serine/threonine kinase (Baso_ST_kin)	S74	<u>L</u> RFLSKI <u>s</u> EYRH <u>Y</u> N <u>Y</u>	2.2882	<u>PRKCZ</u>
<u>0.600</u>	4.365%	Protein Kinase A (PKA_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	T11	<u>D</u> TEEKPR <u>t</u> LHD <u>LC</u> Q <u>A</u>	1.8771	<u>PRKACG</u>

<u>0.494</u>	1.241%	Protein Kinase A (PKA_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	T147	<u>WRSRRREtAL****</u> *_	1.9269	<u>PRKACG</u>
<u>0.563</u>	1.937%	DNA PK (DNA_PK)	DNA damage kinase group (DNA_dam_kin)	T87	<u>NYSVYGNtLEQTV</u> <u>KK</u>	1.3519	<u>PRKDC</u>
<u>0.661</u>	1.338%	PKD1 Binding (PKD1_Bind)	Kinase binding site group (Kin_bind)	D44	<u>LQRSEVYdFAFAD</u> <u>LT</u>	0.3371	<u>PDPK1</u>
<u>0.618</u>	4.823%	14-3-3 Mode 1 (1433_m1)	Phosphoserine/threonine binding group (pST_bind)	T11	<u>DTEEKPRtLHDLc</u> <u>QA</u>	1.8771	<u>YWHAZ</u>
<u>0.694</u>	3.107%	p85 SH3 mode2 (p85_SH3_m 2)	Src homology 3 group (SH3)	P112	<u>ICQRPLCpQEKKR</u> <u>HV</u>	0.9396	<u>PIK3R1</u>
<u>0.576</u>	4.021%	Lck Kinase (Lck_Kin)	Tyrosine kinase group (Y_kin)	Y43	<u>PLQRSEVYDFAFA</u> <u>DL</u>	0.6743	<u>LCK</u>
<u>0.578</u>	2.935%	Src Kinase (Src_Kin)	Tyrosine kinase group (Y_kin)	Y43	<u>PLQRSEVYDFAFA</u> <u>DL</u>	0.6743	<u>SRC</u>

HPV-35 E6 (Alpha-9)





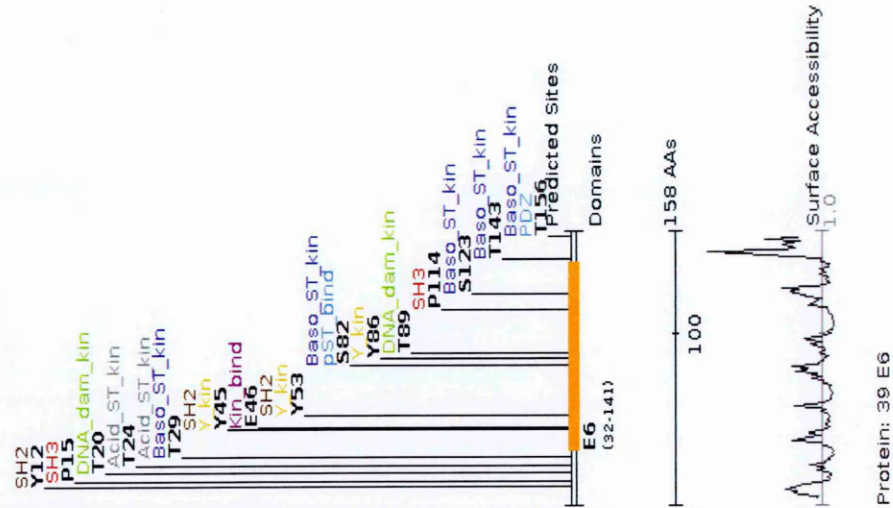
Score	Percentile	Motif	Motifgroup	Site	Sequence	Surface Accessibility	Gene Info
<u>0.381</u>	1.658%	PLK1 Kinase (PLK1)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	S22	<u>LCEVLEE</u> sVH <u>EIRLQ</u>	1.6111	<u>PLK1</u>
<u>0.337</u>	0.883%	PLK1 Kinase (PLK1)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	T100	<u>KKPLSEI</u> HRCII <u>CQ</u>	0.2955	<u>PLK1</u>
<u>0.705</u>	3.307%	AMP_Kinase (AMPK)	Basophilic serine/threonine kinase (Baso_ST_kin)	T11	<u>DPATRPR</u> tLHE <u>LCEV</u>	1.9466	<u>PRKAA1</u>
<u>0.683</u>	3.394%	Akt Kinase (Akt_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	T11	<u>DPATRPR</u> tLHE <u>LCEV</u>	1.9466	<u>AKT1</u>
<u>0.700</u>	4.047%	Akt Kinase (Akt_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	T146	<u>CWRPRPV</u> tQV <u>*****</u>	1.0059	<u>AKT1</u>
<u>0.584</u>	1.452%	Aurora A (AuroA)	Basophilic serine/threonine kinase (Baso_ST_kin)	S82	<u>EYRHYOY</u> sLY <u>GKTLE</u>	1.0657	<u>AURKA</u>
<u>0.628</u>	2.306%	Aurora A (AuroA)	Basophilic serine/threonine kinase (Baso_ST_kin)	S137	<u>GRWTGRC</u> sEC <u>WRPRP</u>	0.3147	<u>AURKA</u>
<u>0.606</u>	1.317%	Aurora B (AuroB)	Basophilic serine/threonine kinase (Baso_ST_kin)	T133	<u>HNIMGRW</u> tGR <u>CSECW</u>	0.7077	<u>AURKB</u>
<u>0.591</u>	1.160%	Aurora B (AuroB)	Basophilic serine/threonine kinase (Baso_ST_kin)	S137	<u>GRWTGRC</u> sEC <u>WRPRP</u>	0.3147	<u>AURKB</u>
<u>0.577</u>	3.655%	Calmodulin dependent Kinase 2 (Cam_Kin2)	Basophilic serine/threonine kinase (Baso_ST_kin)	S82	<u>EYRHYOY</u> sLY <u>GKTLE</u>	1.0657	<u>CAMK2G</u>
<u>0.560</u>	4.381%	PKC zeta (PKC_zeta)	Basophilic serine/threonine kinase (Baso_ST_kin)	S74	<u>LRFLSKI</u> sEYR <u>HYQY</u>	2.2882	<u>PRKCZ</u>

<u>0.547</u>	3.399%	PKC zeta (PKC_zeta)	Basophilic serine/threonine kinase (Baso_ST_kin)	S82	<u>EYRHYQYsLY</u> <u>GKtLE</u>	1.0657	<u>PRKCZ</u>
<u>0.499</u>	1.317%	Protein Kinase A (PKA_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	T11	<u>DPATRPRtLHE</u> <u>LCEV</u>	1.9466	<u>PRKACG</u>
<u>0.624</u>	4.623%	DNA PK (DNA_PK)	DNA damage kinase group (DNA_dam_kin)	T87	<u>OYSLYGKtLE</u> <u>ERVKK</u>	1.6190	<u>PRKDC</u>
<u>0.733</u>	3.996%	Erk D-domain (ErkDD)	Kinase binding site group (Kin_bind)	I99	<u>VKKPLSEiTiR</u> <u>CIIC</u>	0.7387	<u>MAPK1</u>
<u>0.768</u>	1.908%	PIP3-binding PH (PIP3_PH)	Lipid binding group (Lip_bind)	W14 0	<u>TGRSECwRP</u> <u>RPVTQ</u>	1.3269	<u>PIP3-E</u>
<u>0.639</u>	1.768%	PDZ (nNOS) class 1 (PDZ_nNOS_ 1)	PDZ domain binding group (PDZ)	T146	<u>CWRPRPVtOV</u> <u>*****</u>	1.0059	<u>NOS1</u>
<u>0.467</u>	1.113%	14-3-3 Mode 1 (1433_m1)	Phosphoserine/threonine binding group (pST_bind)	T11	<u>DPATRPRtLHE</u> <u>LCEV</u>	1.9466	<u>YWHAZ</u>
<u>0.498</u>	1.587%	CDK1 motif 1 - [ST]Px[KR]x (CDK1_1)	Proline-dependent serine/threonine kinase group (Pro_ST_kin)	T108	<u>IRCIICotPLCP</u> <u>EEK</u>	0.2099	<u>CDK1</u>
<u>0.469</u>	1.375%	CDK1 motif 2 -[ST]PxxK (CDK1_2)	Proline-dependent serine/threonine kinase group (Pro_ST_kin)	T108	<u>IRCIICotPLCP</u> <u>EEK</u>	0.2099	<u>CDK1</u>
<u>0.521</u>	4.661%	Fgr Kinase (Fgr_Kin)	Tyrosine kinase group (Y_kin)	Y43	<u>ELQRREVvKF</u> <u>LFTDL</u>	0.6592	<u>FGR</u>
<u>0.552</u>	2.815%	Lck Kinase (Lck_Kin)	Tyrosine kinase group (Y_kin)	Y43	<u>ELQRREVvKF</u> <u>LFTDL</u>	0.6592	<u>LCK</u>



<u>0.599</u>	4.029%	Src Kinase (Src_Kin)	Tyrosine kinase group (Y_kin)	Y43	<u>ELQRRREV<sub>YKF</sub></u> <u>LF<sub>TDL</sub></u>	0.6592	<u>SRC</u>
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HPV-39 E6 (Alpha-7)

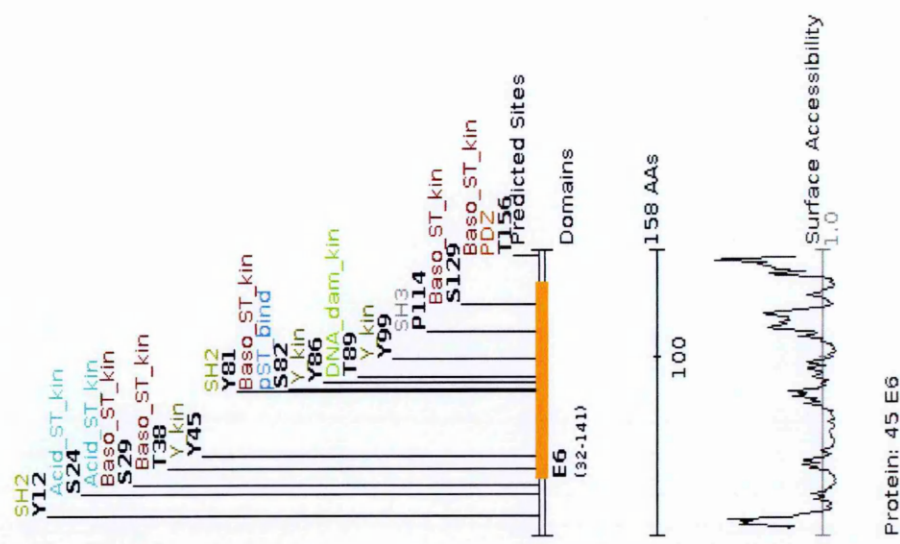


Score	Percentile	Motif	Motifgroup	Site	Sequence	Surface Accessibility	Gene Info
<u>0.279</u>	0.281%	PLK1 Kinase (PLK1)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	T24	<u>LCTTLDtLtQD</u> <u>ITIA</u>	1.9012	<u>PLK1</u>
<u>0.316</u>	0.612%	PLK1 Kinase (PLK1)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	T29	<u>DTTLODItIAC</u> <u>VYCR</u>	0.1470	<u>PLK1</u>
<u>0.684</u>	2.477%	AMP Kinase (AMPK)	Basophilic serine/threonine kinase (Baso_ST_kin)	S123	<u>EKLRLHNLsKR</u> <u>RFHKL</u>	3.2448	<u>PRKAA1</u>
<u>0.589</u>	4.480%	Calmodulin dependent Kinase 2 (Cam_Kin2)	Basophilic serine/threonine kinase (Baso_ST_kin)	S82	<u>IRELRYYSDSV</u> <u>YATT</u>	1.2524	<u>CAMK2G</u>
<u>0.450</u>	0.908%	PKC alpha/beta/gamma (PKC_common)	Basophilic serine/threonine kinase (Baso_ST_kin)	S123	<u>EKLRLHNLsKR</u> <u>RFHKL</u>	3.2448	<u>PRKCA</u>
<u>0.417</u>	0.468%	PKC alpha/beta/gamma (PKC_common)	Basophilic serine/threonine kinase (Baso_ST_kin)	T143	<u>GOCRRCWtTK</u> <u>REDRR</u>	1.0538	<u>PRKCA</u>
<u>0.467</u>	1.881%	PKC epsilon (PKC_epsilon)	Basophilic serine/threonine kinase (Baso_ST_kin)	T29	<u>DTTLODItIAC</u> <u>VYCR</u>	0.1470	<u>PRKCE</u>
<u>0.507</u>	3.417%	PKC mu (PKC_mu)	Basophilic serine/threonine kinase (Baso_ST_kin)	T29	<u>DTTLODItIAC</u> <u>VYCR</u>	0.1470	<u>PRKCM</u>
<u>0.560</u>	4.381%	PKC zeta (PKC_zeta)	Basophilic serine/threonine kinase (Baso_ST_kin)	S123	<u>EKLRLHNLsKR</u> <u>RFHKL</u>	3.2448	<u>PRKCZ</u>
<u>0.505</u>	1.410%	Protein Kinase A (PKA_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	T156	<u>RRLTRREIQV*</u> <u>****</u>	2.9730	<u>PRKACG</u>
0.602	3.418%	DNA PK	DNA damage kinase group	T20	KLPDLCtLtDT	0.5085	PRKDC

<u>0.609</u>	(DNA_PK)	(DNA_dam_kin)		<u>TL0D</u>		
	DNA PK (DNA_PK)	DNA damage kinase group (DNA_dam_kin)	T89	<u>SDSVYATLEN</u> <u>ITNT</u>	1.1501	<u>PRKDC</u>
<u>0.618</u>	PDK1 Binding (PDK1_Bind)	Kinase binding site group (Kin_bind)	E46	<u>LOOTEVYeFAF</u> <u>SDLY</u>	0.3496	<u>PDPK1</u>
<u>0.339</u>	PDZ class 1 (PDZ_class1)	PDZ domain binding group (PDZ)	T156	<u>RRLTRREIQV*</u> <u>****</u>	2.9730	<u>PDZK1</u>
<u>0.522</u>	14-3-3 Mode 1 (1433_m1)	Phosphoserine/threonine binding group (pST_bind)	S82	<u>IRELRYYSDSV</u> <u>YATT</u>	1.2524	<u>YWHAZ</u>
<u>0.552</u>	Crk SH2 (Crk_SH2)	Src homology 2 group (SH2)	Y12	<u>HNPAPERPVKLP</u> <u>DLCI</u>	2.7733	<u>CRK</u>
<u>0.545</u>	Fgr SH2 (Fgr_SH2)	Src homology 2 group (SH2)	Y45	<u>PLOOTEVvEFA</u> <u>FSDL</u>	0.6993	<u>FGR</u>
<u>0.655</u>	Grb2 SH2 (Grb2_SH2)	Src homology 2 group (SH2)	Y53	<u>EFAFSDLvVVY</u> <u>RDGE</u>	0.4269	<u>GRB2</u>
<u>0.561</u>	Itk SH2 (Itk_SH2)	Src homology 2 group (SH2)	Y45	<u>PLOOTEVvEFA</u> <u>FSDL</u>	0.6993	<u>ITK</u>
<u>0.489</u>	Lck SH2 (Lck_SH2)	Src homology 2 group (SH2)	Y45	<u>PLOOTEVvEFA</u> <u>FSDL</u>	0.6993	<u>LCK</u>
<u>0.624</u>	PLCg SH3 (PLCg_SH3)	Src homology 3 group (SH3)	P15	<u>AERPYNLpDL</u> <u>CTTLD</u>	0.4314	<u>PLCG1</u>
<u>0.685</u>	p85 SH3 mode2 (p85_SH3_m2)	Src homology 3 group (SH3)	P114	<u>CCLKPLCpAE</u> <u>KLRHL</u>	0.5481	<u>PIK3R1</u>
<u>0.559</u>	Abl Kinase (Abl_Kin)	Tyrosine kinase group (Y_kin)	Y86	<u>RYSDSVvAT</u> <u>TLENI</u>	0.7515	<u>ABL1</u>

<u>0.515</u>	4.242%	Fgr Kinase (Fgr_Kin)	Tyrosine kinase group (Y_kin)	Y45	<u>PLQQTEV<sub>y</sub>EFA</u> <u>FSDL</u>	0.6993	<u>FGR</u>
<u>0.503</u>	3.485%	Fgr Kinase (Fgr_Kin)	Tyrosine kinase group (Y_kin)	Y53	<u>EFAFSDL<sub>y</sub>VVY</u> <u>RDGE</u>	0.4269	<u>FGR</u>
<u>0.522</u>	4.858%	Itk Kinase (Itk_Kin)	Tyrosine kinase group (Y_kin)	Y45	<u>PLQQTEV<sub>y</sub>EFA</u> <u>FSDL</u>	0.6993	<u>ITK</u>
<u>0.338</u>	0.105%	Itk Kinase (Itk_Kin)	Tyrosine kinase group (Y_kin)	Y53	<u>EFAFSDL<sub>y</sub>VVY</u> <u>RDGE</u>	0.4269	<u>ITK</u>
<u>0.564</u>	3.366%	Lck Kinase (Lck_Kin)	Tyrosine kinase group (Y_kin)	Y45	<u>PLQQTEV<sub>y</sub>EFA</u> <u>FSDL</u>	0.6993	<u>LCK</u>
<u>0.551</u>	2.750%	Lck Kinase (Lck_Kin)	Tyrosine kinase group (Y_kin)	Y86	<u>RYYSDSV<sub>y</sub>AT</u> <u>TLENl</u>	0.7515	<u>LCK</u>
<u>0.544</u>	1.762%	Src Kinase (Src_Kin)	Tyrosine kinase group (Y_kin)	Y45	<u>PLQQTEV<sub>y</sub>EFA</u> <u>FSDL</u>	0.6993	<u>SRC</u>

HPV-45 E6 (Alpha-7)





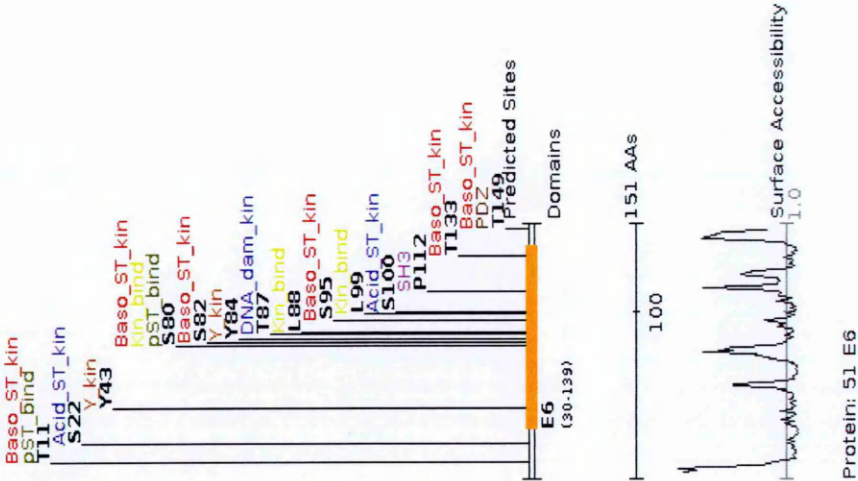
Score	Percentile	Motif	Motifgroup	Site	Sequence	Surface Accessibility	Gene Info
<u>0.340</u>	0.933%	PLK1 Kinase (PLK1)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	S24	<u>LC</u> <u>TELNTs</u> <u>LQDV</u> <u>SIA</u>	1.7654	<u>PLK1</u>
<u>0.266</u>	0.208%	PLK1 Kinase (PLK1)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	S29	<u>NTSLQDV</u> <u>sIACV</u> <u>YCK</u>	0.1445	<u>PLK1</u>
<u>0.667</u>	2.858%	Akt Kinase (Akt_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	S129	<u>KDKRRFHsIAGQ</u> <u>YRG</u>	0.2536	<u>AKT1</u>
<u>0.545</u>	0.633%	Akt Kinase (Akt_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	T156	<u>RLRRRREtOV***</u> <u>**</u> <u>---</u>	2.9730	<u>AKT1</u>
<u>0.602</u>	1.745%	Aurora A (AuroA)	Basophilic serine/threonine kinase (Baso_ST_kin)	S129	<u>KDKRRFHsIAGQ</u> <u>YRG</u>	0.2536	<u>AURKA</u>
<u>0.568</u>	3.218%	Calmodulin dependent Kinase 2 (Cam_Kin2)	Basophilic serine/threonine kinase (Baso_ST_kin)	S82	<u>IRELRYYsNSVY</u> <u>GET</u>	1.2524	<u>CAMK2G</u>
<u>0.536</u>	4.731%	PKC alpha/beta/gamma (PKC_common)	Basophilic serine/threonine kinase (Baso_ST_kin)	T38	<u>ACVYCKA</u> <u>ILERT</u> <u>EVY</u>	1.8691	<u>PRKCA</u>

<u>0.467</u>	1.881%	PKC epsilon (PKC_epsilon)	Basophilic serine/threonine kinase (Baso_ST_kin)	S29	<u>NTSLQDV</u> sIACV <u>YCK</u>	0.1445	<u>PRKCE</u>
<u>0.507</u>	3.417%	PKC mu (PKC_mu)	Basophilic serine/threonine kinase (Baso_ST_kin)	S29	<u>NTSLQDV</u> sIACV <u>YCK</u>	0.1445	<u>PRKCM</u>
<u>0.537</u>	2.593%	PKC zeta (PKC_zeta)	Basophilic serine/threonine kinase (Baso_ST_kin)	S129	<u>KDKRRFH</u> sIAGQ <u>YRG</u>	0.2536	<u>PRKCZ</u>
<u>0.538</u>	2.071%	Protein Kinase A (PKA_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	S129	<u>KDKRRFH</u> sIAGQ <u>YRG</u>	0.2536	<u>PRKACG</u>
<u>0.421</u>	0.619%	Protein Kinase A (PKA_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	T156	<u>RLRRRRE</u> tQV*** <u>**</u>	2.9730	<u>PRKACG</u>
<u>0.579</u>	2.497%	DNA PK (DNA_PK)	DNA damage kinase group (DNA_dam_kin)	T89	<u>SNSVYGE</u> tLEKIT <u>NT</u>	1.6190	<u>PRKDC</u>
<u>0.339</u>	2.347%	PDZ class 1 (PDZ_class1)	PDZ domain binding group (PDZ)	T156	<u>RLRRRRE</u> tQV*** <u>**</u>	2.9730	<u>PDZK1</u>
<u>0.522</u>	1.955%	14-3-3 Mode 1 (1433_m1)	Phosphoserine/threonine binding group (pST_bind)	S82	<u>IRELRY</u> YsNSVY <u>GET</u>	1.2524	<u>YWHAZ</u>
<u>0.528</u>	2.758%	Crk SH2 (Crk_SH2)	Src homology 2 group (SH2)	Y12	<u>DDPKORP</u> yKLDP <u>LCT</u>	2.7733	<u>CRK</u>
<u>0.683</u>	4.044%	Grb2 SH2 (Grb2_SH2)	Src homology 2 group (SH2)	Y81	<u>RIRELRY</u> sNSV <u>YGE</u>	3.3050	<u>GRB2</u>
<u>0.674</u>	2.389%	src SH3 mode2	Src homology 3 group	P114	<u>RCOKPI</u> NnAEK	1.7075	<u>PIK3R1</u>



		(p85_SH3_m2)	(SH3)		<u>RRHL</u>		
<u>0.507</u>	3.792%	Itk Kinase (Itk_Kin)	Tyrosine kinase group (Y_kin)	Y45	<u>TLERTEVYQFAF</u> <u>KDL</u>	0.6993	<u>ITK</u>
<u>0.559</u>	3.151%	Lck Kinase (Lck_Kin)	Tyrosine kinase group (Y_kin)	Y45	<u>TLERTEVYQFAF</u> <u>KDL</u>	0.6993	<u>LCK</u>
<u>0.542</u>	2.385%	Lck Kinase (Lck_Kin)	Tyrosine kinase group (Y_kin)	Y86	<u>RYYSNSVYGETL</u> <u>EKI</u>	0.8834	<u>LCK</u>
<u>0.519</u>	1.619%	Lck Kinase (Lck_Kin)	Tyrosine kinase group (Y_kin)	Y99	<u>KITNTELYNLLIR</u> <u>CL</u>	0.5825	<u>LCK</u>
<u>0.553</u>	1.998%	Src Kinase (Src_Kin)	Tyrosine kinase group (Y_kin)	Y86	<u>RYYSNSVYGETL</u> <u>EKI</u>	0.8834	<u>SRC</u>

HPV-51 E6 (Alpha-5)



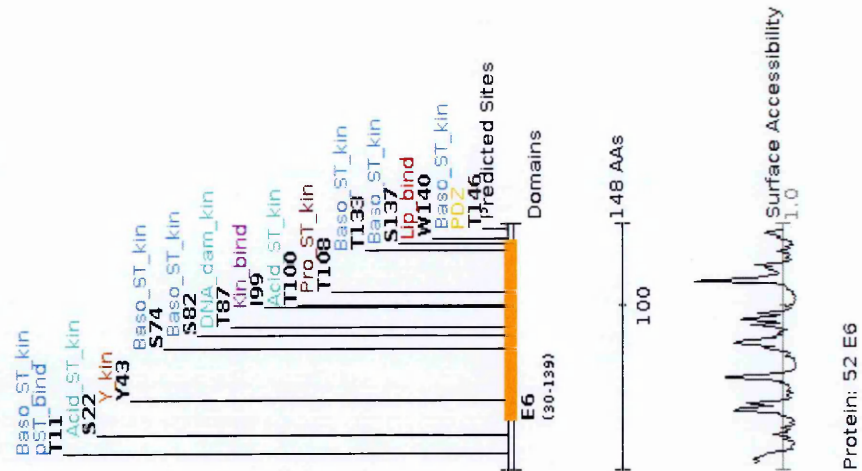
Score	Percentile	Motif	Motifgroup	Site	Sequence	Surface Accessibility	Gene Info
<u>0.304</u>	0.481%	PLK1 Kinase (PLK1)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	S22	<u>LCEALNVsMH</u> <u>NIQVV</u>	0.8560	<u>PLK1</u>
<u>0.343</u>	0.976%	PLK1 Kinase (PLK1)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	S100	<u>KKSLYDLsIRC</u> <u>HRCQ</u>	0.3113	<u>PLK1</u>
<u>0.734</u>	4.906%	AMP_Kinase (AMPK)	Basophilic serine/threonine kinase (Baso_ST_kin)	T11	<u>DKRERPRtLHE</u> <u>LCEA</u>	1.9466	<u>PRKAA1</u>
<u>0.473</u>	0.218%	Akt Kinase (Akt_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	T11	<u>DKRERPRtLHE</u> <u>LCEA</u>	1.9466	<u>AKT1</u>
<u>0.699</u>	4.024%	Akt Kinase (Akt_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	S80	<u>IREYRRYsRSV</u> <u>YGTT</u>	1.8361	<u>AKT1</u>
<u>0.672</u>	3.019%	Akt Kinase (Akt_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	S82	<u>EYRRYSRsVYG</u> <u>TTLE</u>	0.9277	<u>AKT1</u>
<u>0.663</u>	2.748%	Akt Kinase (Akt_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	T149	<u>RTQRNEtQV*</u> <u>****</u>	2.5349	<u>AKT1</u>
<u>0.455</u>	0.294%	Aurora A (AuroA)	Basophilic serine/threonine kinase (Baso_ST_kin)	S80	<u>IREYRRYsRSV</u> <u>YGTT</u>	1.8361	<u>AURKA</u>
<u>0.524</u>	0.606%	Aurora B (AuroB)	Basophilic	S80	<u>IRFYRRYsRSV</u>	1.8361	<u>AURKB</u>

<u>0.657</u>	2.270%	Aurora B (AuroB)	serine/threonine kinase (Baso_ST_kin)	T133	<u>YEIAGRWtGQC</u> <u>ANCW</u>	0.6257	<u>AURKB</u>
<u>0.534</u>	1.796%	Calmodulin dependent Kinase 2 (Cam_Kin2)	Basophilic serine/threonine kinase (Baso_ST_kin)	S80	<u>IREYRRYsRSV</u> <u>YGTT</u>	1.8361	<u>CAMK2G</u>
<u>0.693</u>	0.939%	Clk2 Kinase (Clk2_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	S80	<u>IREYRRYsRSV</u> <u>YGTT</u>	1.8361	<u>CLK2</u>
<u>0.483</u>	2.666%	PKC epsilon (PKC_epsilon)	Basophilic serine/threonine kinase (Baso_ST_kin)	S82	<u>EYRRYSRsVYG</u> <u>TTLE</u>	0.9277	<u>PRKCE</u>
<u>0.525</u>	1.760%	Protein Kinase A (PKA_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	T11	<u>DKRERPRtLHE</u> <u>LCEA</u>	1.9466	<u>PRKACG</u>
<u>0.499</u>	1.319%	Protein Kinase A (PKA_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	S80	<u>IREYRRYsRSV</u> <u>YGTT</u>	1.8361	<u>PRKACG</u>
<u>0.601</u>	4.395%	Protein Kinase A (PKA_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	S95	<u>LEAITKksLYD</u> <u>LSIR</u>	2.6505	<u>PRKACG</u>
<u>0.609</u>	3.790%	DNA PK (DNA_PK)	DNA damage kinase group (DNA_dam_kin)	T87	<u>SRSVYGTtLEAI</u> <u>TKK</u>	0.6815	<u>PRKDC</u>
<u>0.735</u>	4.064%	Erk D-domain (ErkDD)	Kinase binding site group (Kin_bind)	L88	<u>RSVYGTtIEAIT</u> <u>KKS</u>	0.4828	<u>MAPK1</u>

<u>0.721</u>	3.513%	Erk D-domain (ErkDD)	Kinase binding site group (Kin_bind)	L99	<u>TKKSLYDISIRC</u> <u>HRC</u>	0.9099	<u>MAPK1</u>
<u>0.640</u>	1.246%	PKD1 Binding (PKD1_Bind)	Kinase binding site group (Kin_bind)	S80	<u>IREYRRYsRSV</u> <u>YGTT</u>	1.8361	<u>PDPK1</u>
<u>0.339</u>	2.347%	PDZ class 1 (PDZ_class1)	PDZ domain binding group (PDZ)	T149	<u>RTRQRNEIQV*</u> <u>****</u>	2.5349	<u>PDZK1</u>
<u>0.463</u>	1.064%	14-3-3 Mode 1 (1433_m1)	Phosphoserine/threonine binding group (pST_bind)	T11	<u>DKRERPRtLHE</u> <u>LCEA</u>	1.9466	<u>YWHAZ</u>
<u>0.518</u>	1.874%	14-3-3 Mode 1 (1433_m1)	Phosphoserine/threonine binding group (pST_bind)	S80	<u>IREYRRYsRSV</u> <u>YGTT</u>	1.8361	<u>YWHAZ</u>
<u>0.678</u>	2.543%	p85 SH3 mode2 (p85_SH3_m2)	Src homology 3 group (SH3)	P112	<u>RCQRPLGpEEK</u> <u>QKLv</u>	1.7346	<u>PIK3R1</u>
<u>0.609</u>	4.766%	Abl Kinase (Abl_Kin)	Tyrosine kinase group (Y_kin)	Y84	<u>RRYSRSVyGTT</u> <u>LEAI</u>	0.7362	<u>ABL1</u>
<u>0.510</u>	3.919%	Itk Kinase (Itk_Kin)	Tyrosine kinase group (Y_kin)	Y43	<u>ELCRADVvNV</u> <u>AFTEI</u>	0.5573	<u>ITK</u>
<u>0.573</u>	3.827%	Lck Kinase (Lck_Kin)	Tyrosine kinase group (Y_kin)	Y43	<u>ELCRADVvNV</u> <u>AFTEI</u>	0.5573	<u>LCK</u>
<u>0.559</u>	3.130%	Lck Kinase (Lck_Kin)	Tyrosine kinase group (Y_kin)	Y84	<u>RRYSRSVyGTT</u> <u>LEAI</u>	0.7362	<u>LCK</u>



HPV -52 E6 (Alpha-9)



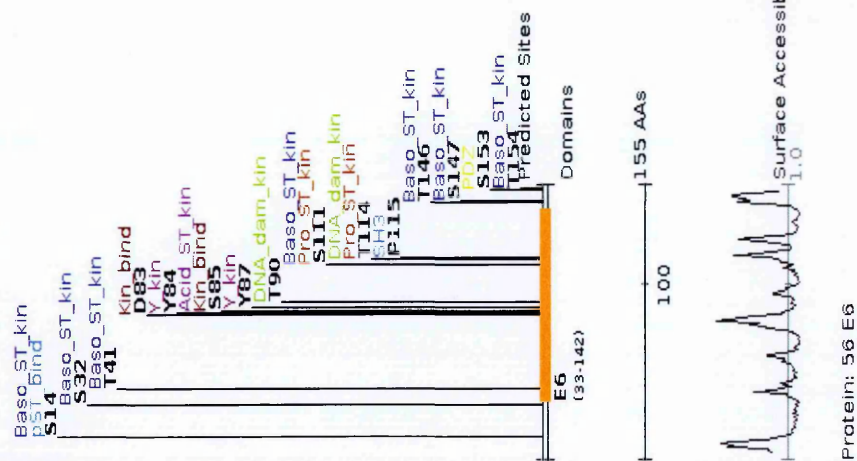
Score	Percentile	Motif	Motifgroup	Site	Sequence	Surface Accessibility	Gene Info
<u>0.381</u>	1.658%	PLK1 Kinase (PLK1)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	S22	<u>LCEVLEE</u> s <u>VHEIR</u> <u>LQ</u>	1.6111	<u>PLK1</u>
<u>0.337</u>	0.883%	PLK1 Kinase (PLK1)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	T100	<u>KKPLSE</u> It <u>IRCIIC</u> <u>Q</u>	0.2955	<u>PLK1</u>
<u>0.705</u>	3.307%	AMP_Kinase (AMPK)	Basophilic serine/threonine kinase (Baso_ST_kin)	T11	<u>DPATRPRtLHEL</u> C <u>EV</u>	1.9466	<u>PRKAA1</u>
<u>0.683</u>	3.394%	Akt Kinase (Akt_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	T11	<u>DPATRPRtLHEL</u> C <u>EV</u>	1.9466	<u>AKT1</u>
<u>0.700</u>	4.047%	Akt Kinase (Akt_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	T146	<u>CWRPRPVtQV</u> *** <u>**</u>	1.0059	<u>AKT1</u>
<u>0.584</u>	1.452%	Aurora A (AuroA)	Basophilic serine/threonine kinase (Baso_ST_kin)	S82	<u>EYRHYQY</u> s <u>LYGK</u> <u>TLE</u>	1.0657	<u>AURKA</u>
<u>0.628</u>	2.306%	Aurora A (AuroA)	Basophilic serine/threonine kinase (Baso_ST_kin)	S137	<u>GRWTGRCs</u> <u>ECW</u> <u>RPRP</u>	0.3147	<u>AURKA</u>
<u>0.606</u>	1.317%	Aurora B (AuroB)	Basophilic serine/threonine kinase (Baso_ST_kin)	T133	<u>HNIMGRWtGRCS</u> <u>ECW</u>	0.7077	<u>AURKB</u>
<u>0.591</u>	1.160%	Aurora B (AuroB)	Basophilic serine/threonine kinase (Baso_ST_kin)	S137	<u>GRWTGRCs</u> <u>ECW</u> <u>RPRP</u>	0.3147	<u>AURKB</u>
<u>0.577</u>	3.655%	Calmodulin dependent Kinase 2 (Cam_Kin2)	Basophilic serine/threonine kinase (Baso_ST_kin)	S82	<u>EYRHYQY</u> s <u>LYGK</u> <u>TLE</u>	1.0657	<u>CAMK2G</u>

<u>0.560</u>	4.381%	PKC zeta (PKC_zeta)	Basophilic serine/threonine kinase (Baso_ST_kin)	S74	<u>LRFLSKIsEYRHY</u> <u>QY</u>	2.2882	<u>PRKCZ</u>
<u>0.547</u>	3.399%	PKC zeta (PKC_zeta)	Basophilic serine/threonine kinase (Baso_ST_kin)	S82	<u>EYRHYQYsLYGK</u> <u>TLE</u>	1.0657	<u>PRKCZ</u>
<u>0.499</u>	1.317%	Protein Kinase A (PKA_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	T11	<u>DPATRPRtLHEL</u> <u>EV</u>	1.9466	<u>PRKACG</u>
<u>0.624</u>	4.623%	DNA PK (DNA_PK)	DNA damage kinase group (DNA_dam_kin)	T87	<u>QYSLYGKtLEER</u> <u>VKK</u>	1.6190	<u>PRKDC</u>
<u>0.733</u>	3.996%	Erk D- domain (ErkDD)	Kinase binding site group (Kin_bind)	I99	<u>VKKPLSEiTIRCII</u> <u>C</u>	0.7387	<u>MAPK1</u>
<u>0.768</u>	1.908%	PIP3-binding PH (PIP3_PH)	Lipid binding group (Lip_bind)	W140	<u>TGRSECwRPRP</u> <u>VTQ</u>	1.3269	<u>PIP3-E</u>
<u>0.639</u>	1.768%	PDZ (nNOS) class I (PDZ_nNOS _1)	PDZ domain binding group (PDZ)	T146	<u>CWRPRPVtQV***</u> <u>**</u>	1.0059	<u>NOS1</u>
<u>0.467</u>	1.113%	I4-3-3 Mode 1 (I433_m1)	Phosphoserine/threonine binding group (pST_bind)	T11	<u>DPATRPRtLHEL</u> <u>EV</u>	1.9466	<u>YWHAZ</u>
<u>0.498</u>	1.587%	CDK1 motif 1 - [ST]Px[KR]x (CDK1_1)	Proline-dependent serine/threonine kinase group (Pro_ST_kin)	T108	<u>IRCIIICQPLCP</u> <u>EE</u> <u>K</u>	0.2099	<u>CDK1</u>
<u>0.469</u>	1.375%	CDK1 motif 2 - [ST]PxxK	Proline-dependent serine/threonine kinase	T108	<u>IRCIIICQPLCP</u> <u>EE</u> <u>K</u>	0.2099	<u>CDK1</u>



	(CDK1_2)	group (Pro_ST_kin)			
<u>0.521</u>	Fgr Kinase (Fgr_Kin)	Tyrosine kinase group (Y_kin)	Y43	<u>ELQRREVYKFLF</u> <u>TDL</u>	<u>FGR</u>  0.6592
<u>0.552</u>	Lck Kinase (Lck_Kin)	Tyrosine kinase group (Y_kin)	Y43	<u>ELQRREVYKFLF</u> <u>TDL</u>	<u>LCK</u>  0.6592
<u>0.599</u>	Src Kinase (Src_Kin)	Tyrosine kinase group (Y_kin)	Y43	<u>ELQRREVYKFLF</u> <u>TDL</u>	<u>SRC</u>  0.6592

HPV-56 E6 (Alpha-6)



Score	Percentile	Motif	Motifgroup	Site	Sequence	Surface Accessibility	Gene Info
<u>0.497</u>	4.406%	Casein Kinase I (Casn_KinI)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	S85	<u>KYRYDYsVYG</u> <u>ATLE</u>	0.9249	<u>CSNK1G2</u>
<u>0.443</u>	2.990%	PLK1 Kinase (PLK1)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	S85	<u>KYRYDYsVYG</u> <u>ATLE</u>	0.9249	<u>PLK1</u>
<u>0.632</u>	1.120%	AMP Kinase (AMPK)	Basophilic serine/threonine kinase (Baso_ST_kin)	S14	<u>NPOERPRsLHHLS</u> <u>EV</u>	1.4202	<u>PRKAA1</u>
<u>0.669</u>	2.928%	Akt Kinase (Akt_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	S14	<u>NPOERPRsLHHLS</u> <u>EV</u>	1.4202	<u>AKT1</u>
<u>0.573</u>	0.935%	Akt Kinase (Akt_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	T154	<u>SREPRESV*****</u> <u>* _</u>	2.4216	<u>AKT1</u>
<u>0.584</u>	1.445%	Aurora A (AuroA)	Basophilic serine/threonine kinase (Baso_ST_kin)	S32	<u>PLIDLRLsCVYCK</u> <u>KE</u>	0.3092	<u>AURKA</u>

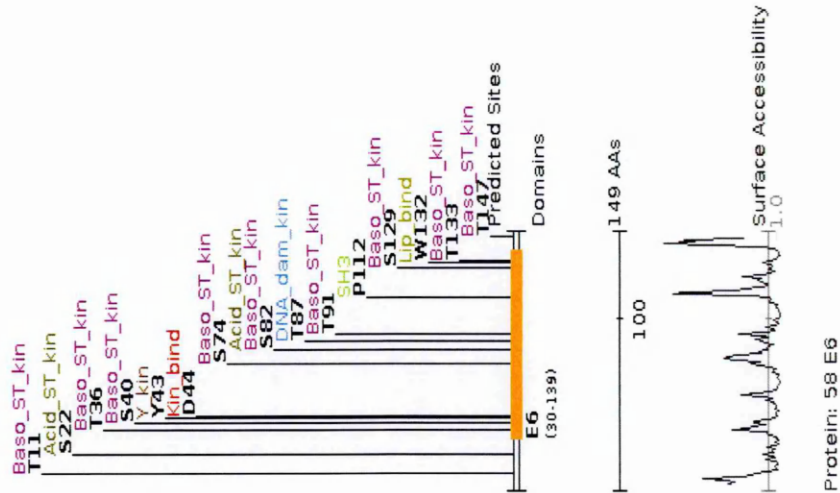
<u>0.577</u>	1.034%	Aurora B (AuroB)	Basophilic serine/threonine kinase (Baso_ST_kin)	S32	<u>PLIDLRLsCVYCK</u> <u>KE</u>	0.3092	<u>AURKB</u>
<u>0.628</u>	1.627%	Aurora B (AuroB)	Basophilic serine/threonine kinase (Baso_ST_kin)	T146	<u>CLGCWRQTSREPR</u> <u>RES</u>	5.0995	<u>AURKB</u>
<u>0.491</u>	0.809%	Calmodulin dependent Kinase 2 (Cam_Kin2)	Basophilic serine/threonine kinase (Baso_ST_kin)	S147	<u>LGCWRQTSREPR</u> <u>EST</u>	4.0259	<u>CAMK2G</u>
<u>0.843</u>	4.906%	Clk2 Kinase (Clk2_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	S14	<u>NPOERPRsLHHLS</u> <u>EV</u>	1.4202	<u>CLK2</u>
<u>0.824</u>	3.986%	Clk2 Kinase (Clk2_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	S111	<u>IRCYRCQsPLTPE</u> <u>EK</u>	0.5247	<u>CLK2</u>
<u>0.743</u>	1.631%	Clk2 Kinase (Clk2_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	S147	<u>LGCWRQTSREPR</u> <u>EST</u>	4.0259	<u>CLK2</u>
<u>0.451</u>	1.153%	PKC epsilon (PKC_epsilon)	Basophilic serine/threonine kinase (Baso_ST_kin)	T41	<u>VYCKKELRAEV</u> <u>YNF</u>	1.6186	<u>PRKCE</u>
<u>0.582</u>	3.561%	Protein Kinase A (PKA_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	S14	<u>NPOERPRsLHHLS</u> <u>EV</u>	1.4202	<u>PRKACG</u>
<u>0.610</u>	4.380%	ATM Kinase (ATM_Kin)	DNA damage kinase group (DNA_dam_kin)	T90	<u>DYSVYGATLESIT</u> <u>KK</u>	0.6328	<u>ATM</u>
<u>0.596</u>	3.552%	ATM Kinase	DNA damage kinase	T114	<u>YRCOSPItPFFKO</u>	1.9559	<u>ATM</u>

<u>0.552</u>	1.617%	(ATM_Kin)	group (DNA_dam_kin)		LH		
		DNA PK (DNA_PK)	DNA damage kinase group (DNA_dam_kin)	T90	<u>DYSVYGatLESIT</u> <u>KK</u>	0.6328	<u>PRKDC</u>
<u>0.240</u>	0.040%	PK1 Binding (PK1_Bind)	Kinase binding site group (Kin_bind)	D83	<u>VRKYRYdYSV</u> <u>YGAT</u>	1.4644	<u>PDPK1</u>
<u>0.602</u>	0.926%	PK1 Binding (PK1_Bind)	Kinase binding site group (Kin_bind)	S85	<u>KYRYDYsVYG</u> <u>ATLE</u>	0.9249	<u>PDPK1</u>
<u>0.339</u>	2.347%	PDZ class 1 (PDZ_class1)	PDZ domain binding group (PDZ)	S153	<u>TSREPREsTV****</u> <u>* _</u>	2.3005	<u>PDZK1</u>
<u>0.491</u>	1.444%	14-3-3 Mode 1 (1433_m1)	Phosphoserine/threonine e binding group (pST_bind)	S14	<u>NPOERPRsLHHLS</u> <u>EV</u>	1.4202	<u>YWHAZ</u>
<u>0.424</u>	0.799%	CDK1 motif 1 - [ST]Px[KR]x (CDK1_1)	Proline-dependent serine/threonine kinase group (Pro_ST_kin)	S111	<u>IRCYRCOsPLTPE</u> <u>EK</u>	0.5247	<u>CDK1</u>
<u>0.392</u>	0.478%	CDK1 motif 2 - [ST]PxxK (CDK1_2)	Proline-dependent serine/threonine kinase group (Pro_ST_kin)	S111	<u>IRCYRCOsPLTPE</u> <u>EK</u>	0.5247	<u>CDK1</u>
<u>0.627</u>	4.402%	CDK1 motif 2 - [ST]PxxK (CDK1_2)	Proline-dependent serine/threonine kinase group (Pro_ST_kin)	T114	<u>YRCOSPLtPEEKQ</u> <u>LH</u>	1.9559	<u>CDK1</u>
<u>0.573</u>	1.921%	Cdc2 Kinase (Cdc2_Kin)	Proline-dependent serine/threonine kinase group (Pro_ST_kin)	T114	<u>YRCOSPLtPEEKQ</u> <u>LH</u>	1.9559	<u>CDC2</u>
<u>0.565</u>	2.535%	Cdk5 Kinase (Cdk5_Kin)	Proline-dependent serine/threonine kinase group (Pro_ST_kin)	T114	<u>YRCOSPLtPEEKQ</u> <u>LH</u>	1.9559	<u>CDK5</u>



<u>0.619</u>	3.293%	Erk1 Kinase (Erk1_Kin)	Proline-dependent serine/threonine kinase group (Pro_ST_kin)	T114	<u>YRCQSPLtPEEKQ</u> <u>LH</u>	1.9559	<u>MAPK3</u>
<u>0.730</u>	4.875%	p85 SH3 mode2 (p85_SH3_m2)	Src homology 3 group (SH3)	P115	<u>RCQSPLTpEEKQ</u> <u>LHC</u>	2.5297	<u>PIK3R1</u>
<u>0.605</u>	4.552%	Abl Kinase (Abl_Kin)	Tyrosine kinase group (Y_kin)	Y84	<u>RKYRYYDySVY</u> <u>GATL</u>	1.4644	<u>ABL1</u>
<u>0.559</u>	2.429%	Abl Kinase (Abl_Kin)	Tyrosine kinase group (Y_kin)	Y87	<u>RYDYSVyGATL</u> <u>ESI</u>	0.5153	<u>ABL1</u>
<u>0.669</u>	2.085%	Insulin Receptor Kinase (InsR_Kin)	Tyrosine kinase group (Y_kin)	Y84	<u>RKYRYYDySVY</u> <u>GATL</u>	1.4644	<u>INSR</u>
<u>0.587</u>	4.725%	Lck Kinase (Lck_Kin)	Tyrosine kinase group (Y_kin)	Y87	<u>RYDYSVyGATL</u> <u>ESI</u>	0.5153	<u>LCK</u>

HPV-58 E6 (Alpha-9)

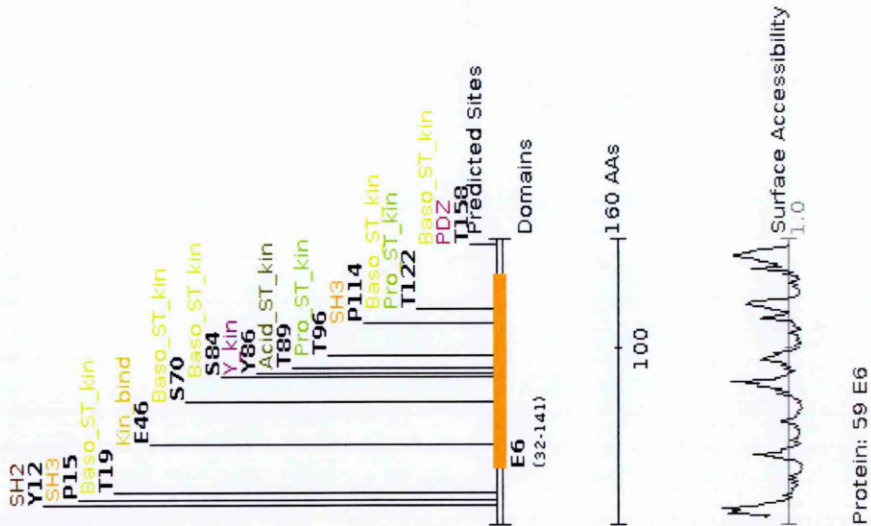


Score	Percentile	Motif	Motifgroup	Site	Sequence	Surface Accessibility	Gene Info
<u>0.315</u>	0.601%	PLK1 Kinase (PLK1)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	S22	<u>LQOALETsV</u> <u>HEIELK</u>	1.3425	<u>PLK1</u>
<u>0.387</u>	1.787%	PLK1 Kinase (PLK1)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	S82	<u>EYRHYNYS</u> <u>LYGDTLE</u>	1.0276	<u>PLK1</u>
<u>0.637</u>	2.508%	Aurora A (AuroA)	Basophilic serine/threonine kinase (Baso_ST_kin)	S82	<u>EYRHYNYS</u> <u>LYGDTLE</u>	1.0276	<u>AURKA</u>
<u>0.694</u>	3.337%	Aurora B (AuroB)	Basophilic serine/threonine kinase (Baso_ST_kin)	T133	<u>HNISGRWtG</u> <u>RCAVCW</u>	0.7077	<u>AURKB</u>
<u>0.510</u>	3.008%	PKC alpha/beta/gamma (PKC_common)	Basophilic serine/threonine kinase (Baso_ST_kin)	T36	<u>KCVECKtL</u> <u>QRSEVY</u>	3.7001	<u>PRKCA</u>
<u>0.529</u>	4.077%	PKC alpha/beta/gamma (PKC_common)	Basophilic serine/threonine kinase (Baso_ST_kin)	T91	<u>YGDTLEQIL</u> <u>KKCLNE</u>	3.2717	<u>PRKCA</u>
<u>0.475</u>	1.526%	PKC alpha/beta/gamma (PKC_common)	Basophilic serine/threonine kinase (Baso_ST_kin)	S129	<u>NKRFHNIsg</u> <u>RWTGRC</u>	0.7327	<u>PRKCA</u>
<u>0.517</u>	3.998%	PKC mu (PKC_mu)	Basophilic serine/threonine kinase (Baso_ST_kin)	S74	<u>LRLSKIsEY</u> <u>RHYNY</u>	2.2882	<u>PRKCM</u>
0.560	4.381%	PKC zeta (PKC_zeta)	Basophilic	S40	<u>CKKTIORsF</u>	2.0981	PRKCZ



<u>0.609</u>	4.788%	Protein Kinase A (PKA_Kin)	serine/threonine kinase (Baso_ST_kin)	T11	<u>DAEEKPRtL</u> <u>HDLCOA</u>	1.8771	<u>PRKACG</u>
<u>0.489</u>	1.185%	Protein Kinase A (PKA_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	T147	<u>WRPRRRQt</u> <u>QV*****</u>	2.9730	<u>PRKACG</u>
<u>0.515</u>	0.925%	DNA PK (DNA_PK)	DNA damage kinase group (DNA_dam_kin)	T87	<u>NYSLYGDtL</u> <u>EOtLKK</u>	1.3519	<u>PRKDC</u>
<u>0.682</u>	1.379%	PDK1 Binding (PDK1_Bind)	Kinase binding site group (Kin_bind)	D44	<u>LORSEVYdF</u> <u>VFADLR</u>	0.2477	<u>PDPK1</u>
<u>0.813</u>	3.682%	PIP3-binding PH (PIP3_PH)	Lipid binding group (Lip_bind)	W132	<u>FHNISGRwT</u> <u>GRCaVC</u>	1.3065	<u>PIP3-E</u>
<u>0.694</u>	3.107%	p85 SH3 mode2 (p85_SH3_m2)	Src homology 3 group (SH3)	P112	<u>ICQRPLCpQ</u> <u>EKKRHV</u>	0.9396	<u>PIK3R1</u>
<u>0.517</u>	4.388%	Fgr Kinase (Fgr_Kin)	Tyrosine kinase group (Y_kin)	Y43	<u>TLORSEVvD</u> <u>FVFADL</u>	0.4954	<u>FGR</u>
<u>0.706</u>	3.674%	Insulin Receptor Kinase (InsR_Kin)	Tyrosine kinase group (Y_kin)	Y43	<u>TLORSEVvD</u> <u>FVFADL</u>	0.4954	<u>INSR</u>
<u>0.504</u>	3.600%	Itk Kinase (Itk_Kin)	Tyrosine kinase group (Y_kin)	Y43	<u>TLORSEVvD</u> <u>FVFADL</u>	0.4954	<u>ITK</u>
<u>0.496</u>	1.079%	Lck Kinase (Lck_Kin)	Tyrosine kinase group (Y_kin)	Y43	<u>TLORSEVvD</u> <u>FVFADL</u>	0.4954	<u>LCK</u>
<u>0.531</u>	1.422%	Src Kinase (Src_Kin)	Tyrosine kinase group (Y_kin)	Y43	<u>TLORSEVvD</u> <u>FVFADL</u>	0.4954	<u>SRC</u>

HPV-59 E6 (Alpha-7)



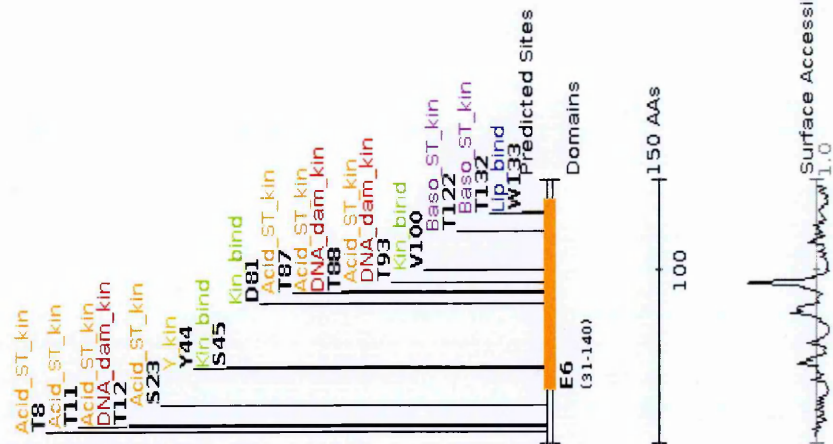
Score	Percentile	Motif	Motifgroup	Site	Sequence	Surface Accessibility	Gene Info
<u>0.541</u>	2.336%	Casein Kinase 2 (Casn_Kin2)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	T89	<u>RDSVYGEtLE</u> <u>AETKT</u>	0.8178	<u>CSNK2B</u>
<u>0.712</u>	3.667%	AMP Kinase (AMPK)	Basophilic serine/threonine kinase (Baso_ST_kin)	T19	<u>YKLPLDLStTLN</u> <u>IPLH</u>	0.7265	<u>PRKAA1</u>
<u>0.616</u>	1.589%	Akt Kinase (Akt_Kin)	Basophilic serine/threonine kinase (Baso_ST_kin)	T158	<u>QRQARSEtLV*</u> <u>****</u>	0.9686	<u>AKT1</u>
<u>0.608</u>	1.856%	Aurora A (AuroA)	Basophilic serine/threonine kinase (Baso_ST_kin)	S70	<u>AACLKCIstFYA</u> <u>RVRE</u>	0.1582	<u>AURKA</u>
<u>0.513</u>	0.607%	Aurora A (AuroA)	Basophilic serine/threonine kinase (Baso_ST_kin)	S84	<u>ELRYYRDsVY</u> <u>GETLE</u>	1.1561	<u>AURKA</u>
<u>0.657</u>	2.257%	Aurora B (AuroB)	Basophilic serine/threonine kinase (Baso_ST_kin)	S84	<u>ELRYYRDsVY</u> <u>GETLE</u>	1.1561	<u>AURKB</u>
<u>0.529</u>	4.077%	PKC alpha/beta/gamma (PKC_common)	Basophilic serine/threonine kinase (Baso_ST_kin)	T122	<u>TDKCLKHItEKR</u> <u>RFHN</u>	2.1400	<u>PRKCA</u>
<u>0.466</u>	2.913%	PKC delta (PKC_delta)	Basophilic serine/threonine kinase (Baso_ST_kin)	S70	<u>AACLKCIstFYA</u> <u>RVRE</u>	0.1582	<u>PRKCD</u>
0.499	3.746%	PKC epsilon	Basophilic	S84	<u>ELRYYRDsVY</u>	1.1561	<u>PRKCE</u>

	(PKC_epsilon)	serine/threonine kinase (Baso_ST_kin)	GETLE		
<u>0.589</u>	3.851%	Protein Kinase A (PKA_Kin)	S84	<u>ELRYYRDSVY</u> <u>GETLE</u>	<u>1.1561</u> <u>PRKACG</u>
<u>0.613</u>	1.056%	PDK1 Binding (PDK1_Bind)	E46	<u>LQEREVFeFAF</u> <u>NDLF</u>	<u>0.1932</u> <u>PDPK1</u>
<u>0.614</u>	1.335%	PDZ (nNOS) class 1 (PDZ_nNOS_1)	T158	<u>QROARSEtLV*</u> <u>****</u>	<u>0.9686</u> <u>NOS1</u>
<u>0.362</u>	3.363%	PDZ class 1 (PDZ_class1)	T158	<u>QROARSEtLV*</u> <u>****</u>	<u>0.9686</u> <u>PDZK1</u>
<u>0.575</u>	3.028%	CDK1 motif 1 - [ST]Px[KR]x (CDK1_1)	T96	<u>TLEAETKtPLH</u> <u>ELLJ</u>	<u>1.6563</u> <u>CDK1</u>
<u>0.551</u>	2.947%	CDK1 motif 2 - [ST]PxxK (CDK1_2)	T96	<u>TLEAETKtPLH</u> <u>ELLJ</u>	<u>1.6563</u> <u>CDK1</u>
<u>0.566</u>	1.761%	Cdc2 Kinase (Cdc2_Kin)	T96	<u>TLEAETKtPLH</u> <u>ELLJ</u>	<u>1.6563</u> <u>CDC2</u>
<u>0.487</u>	1.017%	Cdk5 Kinase (Cdk5_Kin)	T96	<u>TLEAETKtPLH</u> <u>ELLJ</u>	<u>1.6563</u> <u>CDK5</u>
<u>0.620</u>	4.242%	Cdk5 Kinase (Cdk5_Kin)	T122	<u>TDKCLKHtEKR</u> <u>RFHN</u>	<u>2.1400</u> <u>CDK5</u>
<u>0.489</u>	1.709%	Crk SH2	Y12	<u>EDPTORPvKLP</u>	<u>2.7733</u> <u>CRK</u>

	(Crk_SH2)	(SH2)		DLST	
<u>0.502</u>	PLCg C-terminal SH2 (PLCg_CSH2)	Src homology 2 group (SH2)	Y12	<u>EDPTQRPyKLP</u> <u>DLST</u>	<u>PLCG1</u> 2.7733
<u>0.614</u>	PLCg SH3 (PLCg_SH3)	Src homology 3 group (SH3)	P15	<u>TQRPYKLpDL</u> <u>STTLN</u>	<u>PLCG1</u> 1.0786
<u>0.612</u>	p85 SH3 mode2 (p85_SH3_m2)	Src homology 3 group (SH3)	P114	<u>RCLKPLCpTD</u> <u>KLKHI</u>	<u>PIK3R1</u> 0.7550
<u>0.491</u>	Lck Kinase (Lck_Kin)	Tyrosine kinase group (Y_kin)	Y86	<u>RYYRDSV_yGE</u> <u>TLEAE</u>	<u>LCK</u> 0.8834
<u>0.477</u>	Src Kinase (Src_Kin)	Tyrosine kinase group (Y_kin)	Y86	<u>RYYRDSV_yGE</u> <u>TLEAE</u>	<u>SRC</u> 0.8834



HPV-6 E6 (Alpha-10)

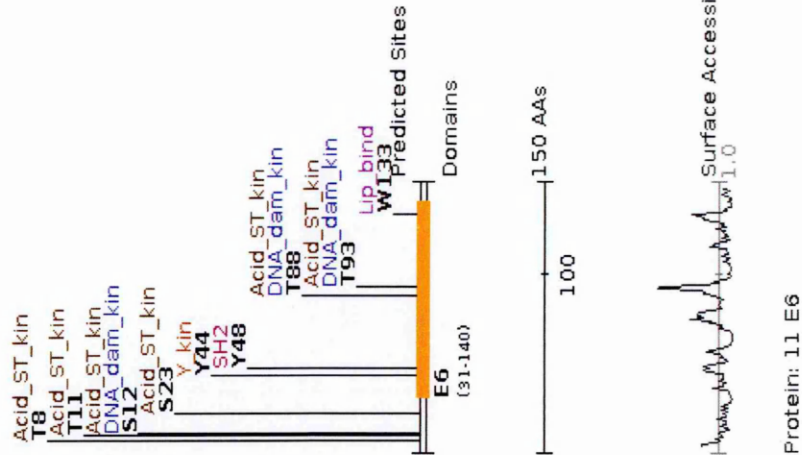


Score	Percentile	Motif	Motifgroup	Site	Sequence	Surface Accessibility	Gene Info
<u>0.378</u>	0.361%	Casein Kinase 1 (Casn_Kin1)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	T11	<u>ANASTSATI</u> <u>DQLCK</u>	0.7565	<u>CSNK1G2</u>
<u>0.472</u>	2.903%	Casein Kinase 1 (Casn_Kin1)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	T12	<u>NASTSATID</u> <u>QLCKT</u>	0.9776	<u>CSNK1G2</u>
<u>0.524</u>	1.790%	Casein Kinase 2 (Casn_Kin2)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	T87	<u>FDYAGYAT</u> <u>VEEETK</u>	0.9712	<u>CSNK2B</u>
<u>0.522</u>	1.740%	Casein Kinase 2 (Casn_Kin2)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	T88	<u>DYAGYAT</u> <u>VEEETKQ</u>	1.0734	<u>CSNK2B</u>
<u>0.549</u>	2.591%	Casein Kinase 2 (Casn_Kin2)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	T93	<u>ATTVEEEK</u> <u>QDILDV</u>	5.7373	<u>CSNK2B</u>
<u>0.579</u>	4.141%	GSK3-improved (GSK3b)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	T8	<u>MESANASIS</u> <u>ATTIDQ</u>	0.8748	<u>GSK3B</u>
<u>0.320</u>	0.661%	PLK1 Kinase (PLK1)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	S23	<u>LCKTFNLsM</u> <u>HTLOIN</u>	0.8220	<u>PLK1</u>
<u>0.519</u>	3.502%	PKC alpha/beta/gamma (PKC_common)	Basophilic serine/threonine kinase (Baso_ST_kin)	T132	<u>RFKLNCTW</u> <u>KGRCLH</u>	0.6161	<u>PRKCA</u>

<u>0.529</u>	4.791%	PKC mu (PKC_mu)	Basophilic serine/threonine kinase (Baso_ST_kin)	T122	<u>EKVVKHILtK</u> <u>ARFIKL</u>	0.7566	<u>PRKCM</u>
<u>0.611</u>	4.508%	ATM Kinase (ATM_Kin)	DNA damage kinase group (DNA_dam_kin)	T88	<u>DYAGYATt</u> <u>VEEETKQ</u>	1.0734	<u>ATM</u>
<u>0.510</u>	0.842%	DNA PK (DNA_PK)	DNA damage kinase group (DNA_dam_kin)	T12	<u>NASTSATtID</u> <u>QLCKt</u>	0.9776	<u>PRKDC</u>
<u>0.593</u>	3.022%	DNA PK (DNA_PK)	DNA damage kinase group (DNA_dam_kin)	T93	<u>ATTVEEEtK</u> <u>QDILDV</u>	5.7373	<u>PRKDC</u>
<u>0.734</u>	4.015%	Erk D-domain (ErkDD)	Kinase binding site group (Kin_bind)	V100	<u>TKQDILDvLI</u> <u>RCYLC</u>	0.2652	<u>MAPK1</u>
<u>0.646</u>	1.284%	PDK1 Binding (PDK1_Bind)	Kinase binding site group (Kin_bind)	S45	<u>LTTAEIYsY</u> <u>AYKHLK</u>	0.8366	<u>PDPK1</u>
<u>0.215</u>	0.036%	PDK1 Binding (PDK1_Bind)	Kinase binding site group (Kin_bind)	D81	<u>INQYRHFdY</u> <u>AGYATT</u>	0.7064	<u>PDPK1</u>
<u>0.776</u>	2.158%	PIP3-binding PH (PIP3_PH)	Lipid binding group (Lip_bind)	W133	<u>FIKLNCTwK</u> <u>GRCLHC</u>	0.7226	<u>PIP3-E</u>
<u>0.495</u>	3.115%	Itk Kinase (Itk_Kin)	Tyrosine kinase group (Y_kin)	Y44	<u>ALTTAEIvS</u> <u>YAYKHL</u>	0.9247	<u>ITK</u>



HPV-11 E6 (Alpha-10)



Score	Percentile	Motif	Motifgroup	Site	Sequence	Surface Accessibility	Gene Info
<u>0.492</u>	4.038%	Casein Kinase 1 (Casn_Kin1)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	T8	<u>MESKDAS</u> <u>tSATS</u> <u>IDQ</u>	0.8748	<u>CSNK1G2</u>
<u>0.308</u>	0.047%	Casein Kinase 1 (Casn_Kin1)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	T11	<u>KDASTA</u> <u>tSIDQ</u> <u>LCK</u>	0.7024	<u>CSNK1G2</u>
<u>0.478</u>	3.152%	Casein Kinase 1 (Casn_Kin1)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	S12	<u>DASTSA</u> <u>tIDQL</u> <u>CKT</u>	0.9077	<u>CSNK1G2</u>
<u>0.474</u>	0.826%	Casein Kinase 2 (Casn_Kin2)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	T88	<u>NYAAYAP</u> <u>tVEE</u> <u>ETNE</u>	1.1501	<u>CSNK2B</u>
<u>0.500</u>	1.241%	Casein Kinase 2 (Casn_Kin2)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	T93	<u>APTVEEE</u> <u>tNEDI</u> <u>LKV</u>	4.7909	<u>CSNK2B</u>
<u>0.552</u>	2.992%	GSK3-improved (GSK3b)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	T8	<u>MESKDAS</u> <u>tSATS</u> <u>IDQ</u>	0.8748	<u>GSK3B</u>
<u>0.324</u>	0.703%	PLK1 Kinase (PLK1)	Acidophilic serine/threonine kinase group (Acid_ST_kin)	S23	<u>LCKTFNL</u> <u>sLHTL</u> <u>QIQ</u>	0.6850	<u>PLK1</u>

<u>0.619</u>	4.993%	ATM Kinase (ATM_Kin)	DNA damage kinase group (DNA_dam_kin)	T88	<u>NYAA<sub>Y</sub>APIVEE</u> <u>ETNE</u>	1.1501	<u>ATM</u>
<u>0.523</u>	1.042%	DNA PK (DNA_PK)	DNA damage kinase group (DNA_dam_kin)	S12	<u>DASTSATsIDOL</u> <u>CKT</u>	0.9077	<u>PRKDC</u>
<u>0.594</u>	3.044%	DNA PK (DNA_PK)	DNA damage kinase group (DNA_dam_kin)	T88	<u>NYAA<sub>Y</sub>APIVEE</u> <u>ETNE</u>	1.1501	<u>PRKDC</u>
<u>0.526</u>	1.088%	DNA PK (DNA_PK)	DNA damage kinase group (DNA_dam_kin)	T93	<u>APTVEEEtNEDI</u> <u>LKV</u>	4.7909	<u>PRKDC</u>
<u>0.835</u>	4.808%	PIP3-binding PH (PIP3_PH)	Lipid binding group (Lip_bind)	W133	<u>FIKLNNQwKGR</u> <u>CLHC</u>	2.7014	<u>PIP3-E</u>
<u>0.677</u>	3.912%	Grb2 SH2 (Grb2_SH2)	Src homology 2 group (SH2)	Y48	<u>AEIYAYAvKNL</u> <u>KVWVW</u>	1.5655	<u>GRB2</u>
<u>0.572</u>	2.906%	Abl Kinase (Abl_Kin)	Tyrosine kinase group (Y_kin)	Y44	<u>ALTTAEIvAYAY</u> <u>KNL</u>	0.6971	<u>ABLJ</u>
<u>0.503</u>	3.550%	Itk Kinase (Itk_Kin)	Tyrosine kinase group (Y_kin)	Y44	<u>ALTTAEIvAYAY</u> <u>KNL</u>	0.6971	<u>ITK</u>
<u>0.562</u>	3.269%	Lck Kinase (Lck_Kin)	Tyrosine kinase group (Y_kin)	Y44	<u>ALTTAEIvAYAY</u> <u>KNL</u>	0.6971	<u>LCK</u>

## REFERENCES

- Adam JL, Briggs MW & McCance DJ (2000) A mutagenic analysis of the E5 protein of human papillomavirus type 16 reveals that E5 binding to the vacuolar H<sup>+</sup>-ATPase is not sufficient for biological activity, using mammalian and yeast expression systems. *Virology* **272**: 315–25
- Affolter A, Drigotas M, Fruth K, Schmidtmann I, Brochhausen C, Mann WJ & Brieger J (2013) Increased radioresistance via G12S K-Ras by compensatory upregulation of MAPK and PI3K pathways in epithelial cancer. *Head & neck* **35**: 220–8
- Agarwal ML, Agarwal A, Taylor WR & Stark GR (1995) p53 controls both the G2/M and the G1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America* **92**: 8493–7
- Agnantis NJ, Spandidos DA, Mahera H, Parissi P, Kakkanas A, Pintzas A & Papacharalampous NX (1988) Immunohistochemical study of ras oncogene expression in endometrial and cervical human lesions. *European journal of gynaecological oncology* **9**: 360–5
- Ahn J-H, McAvoy T, Rakhilin S V, Nishi A, Greengard P & Nairn AC (2007) Protein kinase A activates protein phosphatase 2A by phosphorylation of the B56delta subunit. *Proceedings of the National Academy of Sciences of the United States of America* **104**: 2979–84
- Aitken A (2002) Functional specificity in 14-3-3 isoform interactions through dimer formation and phosphorylation. Chromosome location of mammalian isoforms and variants. *Plant molecular biology* **50**: 993–1010
- Aitken A (2006) 14-3-3 proteins: a historic overview. *Seminars in Cancer Biology* **16**: 162–172
- Aitken A, Collinge DB, van Heusden BP, Isobe T, Roseboom PH, Rosenfeld G & Soll J (1992) 14-3-3 proteins: a highly conserved, widespread family of eukaryotic proteins. *Trends in biochemical sciences* **17**: 498–501
- Alam R, Hachiya N, Sakaguchi M, Kawabata S, Iwanaga S, Kitajima M, Mihara K & Omura T (1994) cDNA cloning and characterization of mitochondrial import stimulation factor (MSF) purified from rat liver cytosol. *Journal of biochemistry* **116**: 416–25
- Alcocer-González JM, Berumen J, Taméz-Guerra R, Bermúdez-Morales V, Peralta-Zaragoza O, Hernández-Pando R, Moreno J, Gariglio P & Madrid-Marina V (2006) In vivo expression of immunosuppressive cytokines in

- human papillomavirus-transformed cervical cancer cells. *Viral immunology* **19**: 481–91
- Alfandari J, Shnitman Magal S, Jackman A, Schlegel R, Gonen P & Sherman L (1999) HPV16 E6 oncoprotein inhibits apoptosis induced during serum-calcium differentiation of foreskin human keratinocytes. *Virology* **257**: 383–96
- Alomari A, Abi-Raad R, Buza N & Hui P (2014) Frequent KRAS mutation in complex mucinous epithelial lesions of the endometrium. *Modern pathology: an official journal of the United States and Canadian Academy of Pathology, Inc* **27**: 675–80
- Alvarez-Salas LM, Cullinan AE, Siwkowski A, Hampel A & DiPaolo JA (1998) Inhibition of HPV-16 E6/E7 immortalization of normal keratinocytes by hairpin ribozymes. *Proc Natl Acad Sci U S A* **95**: 1189–1194
- An J, Mo D, Liu H, Veena MS, Srivatsan ES, Massoumi R & Rettig MB (2008) Inactivation of the CYLD deubiquitinase by HPV E6 mediates hypoxia-induced NF-kappaB activation. *Cancer cell* **14**: 394–407
- Anderson JM (1996) Cell signalling: MAGUK magic. *Current Biology* **6**: 382–384
- Antinore MJ, Birrer MJ, Patel D, Nader L & McCance DJ (1996) The human papillomavirus type 16 E7 gene product interacts with and trans-activates the AP1 family of transcription factors. *The EMBO journal* **15**: 1950–60
- Aplan PD (2006) Causes of oncogenic chromosomal translocation. *Trends in genetics: TIG* **22**: 46–55
- Arany I & Tyring SK (1996) Status of local cellular immunity in interferon-responsive and -nonresponsive human papillomavirus-associated lesions. *Sexually transmitted diseases* **23**: 475–80
- Arbeit JM, Howley PM & Hanahan D (1996) Chronic estrogen-induced cervical and vaginal squamous carcinogenesis in human papillomavirus type 16 transgenic mice. *Proceedings of the National Academy of Sciences of the United States of America* **93**: 2930–5
- Arbeit JM, Munger K, Howley PM & Hanahan D (1993) Neuroepithelial carcinomas in mice transgenic with human papillomavirus type 16 E6/E7 ORFs. *Am J Pathol* **142**: 1187–1197

- Arbeit JM, Münger K, Howley PM & Hanahan D (1994) Progressive squamous epithelial neoplasia in K14-human papillomavirus type 16 transgenic mice. *Journal of virology* **68**: 4358–68
- Aromseree S, Chaiwongkot A, Ekalaksananan T, Kongyingyoes B, Patarapadungkit N & Pientong C (2014) The three most common human papillomavirus oncogenic types and their integration state in Thai women with cervical precancerous lesions and carcinomas. *Journal of medical virology*
- Arora S, Matta A, Shukla NK, Deo S V & Ralhan R (2005) Identification of differentially expressed genes in oral squamous cell carcinoma. *Mol Carcinog* **42**: 97–108
- Arroyo M, Bagchi S & Raychaudhuri P (1993) Association of the human papillomavirus type 16 E7 protein with the S-phase-specific E2F-cyclin A complex. *Molecular and cellular biology* **13**: 6537–46
- Artandi SE & DePinho RA (2010) Telomeres and telomerase in cancer. *Carcinogenesis* **31**: 9–18
- Ashcroft M, Kubbutat MH & Vousden KH (1999) Regulation of p53 function and stability by phosphorylation. *Molecular and cellular biology* **19**: 1751–8
- Astuti P, Boutros R, Ducommun B & Gabrielli B (2010) Mitotic phosphorylation of Cdc25B Ser321 disrupts 14-3-3 binding to the high affinity Ser323 site. *The Journal of biological chemistry* **285**: 34364–70
- Autieri M V & Carbone CJ (1999) 14-3-3Gamma interacts with and is phosphorylated by multiple protein kinase C isoforms in PDGF-stimulated human vascular smooth muscle cells. *DNA and cell biology* **18**: 555–64
- Avvakumov N, Torchia J & Mymryk JS (2003) Interaction of the HPV E7 proteins with the pCAF acetyltransferase. *Oncogene* **22**: 3833–3841
- Bachmair A, Finley D & Varshavsky A (1986) In vivo half-life of a protein is a function of its amino-terminal residue. *Science (New York, N.Y.)* **234**: 179–86
- Badaracco G, Venuti A, Sedati A & Marcante ML (2002) HPV16 and HPV18 in genital tumors: Significantly different levels of viral integration and correlation to tumor invasiveness. *Journal of medical virology* **67**: 574–82

- Bai C, Tang S & Chen X (2014) Quantitative proteomic dissection of a native 14-3-3 $\epsilon$  interacting protein complex associated with hepatocellular carcinoma. *Amino acids* **46**: 841–52
- Baldin V, Cans C, Knibiehler M & Ducommun B (1997) Phosphorylation of Human CDC25B Phosphatase by CDK1-Cyclin A Triggers Its Proteasome-dependent Degradation. *Journal of Biological Chemistry* **272**: 32731–32734
- Balsitis S, Dick F, Dyson N & Lambert PF (2006) Critical roles for non-pRb targets of human papillomavirus type 16 E7 in cervical carcinogenesis. *Cancer Res* **66**: 9393–9400
- Band V, De Caprio JA, Delmolino L, Kulesa V & Sager R (1991) Loss of p53 protein in human papillomavirus type 16 E6-immortalized human mammary epithelial cells. *J Virol* **65**: 6671–6676
- Bañuelos A, Reyes E, Ocadiz R, Alvarez E, Moreno M, Monroy A & Gariglio P (2003) Neocarzinostatin induces an effective p53-dependent response in human papillomavirus-positive cervical cancer cells. *The Journal of pharmacology and experimental therapeutics* **306**: 671–80
- Bao S, Tibbetts RS, Brumbaugh KM, Fang Y, Richardson DA, Ali A, Chen SM, Abraham RT & Wang XF (2001) ATR/ATM-mediated phosphorylation of human Rad17 is required for genotoxic stress responses. *Nature* **411**: 969–74
- Barbosa MS, Edmonds C, Fisher C, Schiller JT, Lowy DR & Vousden KH (1990) The region of the HPV E7 oncoprotein homologous to adenovirus E1a and Sv40 large T antigen contains separate domains for Rb binding and casein kinase II phosphorylation. *The EMBO journal* **9**: 153–60
- Barbosa MS, Lowy DR & Schiller JT (1989) Papillomavirus polypeptides E6 and E7 are zinc-binding proteins. *Journal of virology* **63**: 1404–7
- Barrow-Laing L, Chen W & Roman A (2010) Low- and high-risk human papillomavirus E7 proteins regulate p130 differently. *Virology* **400**: 233–9
- Bassères DS & Baldwin AS (2006) Nuclear factor-kappaB and inhibitor of kappaB kinase pathways in oncogenic initiation and progression. *Oncogene* **25**: 6817–30
- Baumgartner M, Radziwill G, Lorger M, Weiss A & Moelling K (2008) c-Src-mediated epithelial cell migration and invasion regulated by PDZ binding site. *Molecular and cellular biology* **28**: 642–55

- Baxter MK, McPhillips MG, Ozato K & McBride AA (2005) The mitotic chromosome binding activity of the papillomavirus E2 protein correlates with interaction with the cellular chromosomal protein, Brd4. *Journal of virology* **79**: 4806–18
- Bedell MA, Jones KH, Grossman SR & Laimins LA (1989) Identification of human papillomavirus type 18 transforming genes in immortalized and primary cells. *Journal of virology* **63**: 1247–55
- Bergant M & Banks L (2013) SNX17 facilitates infection with diverse papillomavirus types. *Journal of virology* **87**: 1270–3
- Bergant Marušič M, Ozbun MA, Campos SK, Myers MP & Banks L (2012) Human papillomavirus L2 facilitates viral escape from late endosomes via sorting nexin 17. *Traffic (Copenhagen, Denmark)* **13**: 455–67
- Bernat A, Avvakumov N, Mymryk JS & Banks L (2003) Interaction between the HPV E7 oncoprotein and the transcriptional coactivator p300. *Oncogene* **22**: 7871–81
- Bilder D (2000) Cooperative Regulation of Cell Polarity and Growth by Drosophila Tumor Suppressors. *Science* **289**: 113–116
- Bilder D (2004) Epithelial polarity and proliferation control: links from the Drosophila neoplastic tumor suppressors. *Genes & development* **18**: 1909–25
- Bilder D & Perrimon N (2000) Localization of apical epithelial determinants by the basolateral PDZ protein Scribble. *Nature* **403**: 676–80
- Blakaj DM, Fernandez-Fuentes N, Chen Z, Hegde R, Fiser A, Burk RD & Brenowitz M (2009) Evolutionary and biophysical relationships among the papillomavirus E2 proteins. *Frontiers in bioscience (Landmark edition)* **14**: 900–17
- Blanton RA, Perez-Reyes N, Merrick DT & McDougall JK (1991) Epithelial cells immortalized by human papillomaviruses have premalignant characteristics in organotypic culture. *The American journal of pathology* **138**: 673–85
- Blaydes JP, Luciani MG, Pospisilova S, Ball HM, Vojtesek B & Hupp TR (2001) Stoichiometric phosphorylation of human p53 at Ser315 stimulates p53-dependent transcription. *The Journal of biological chemistry* **276**: 4699–708



- Blumer KJ, Johnson GL & Lange-Carter CA (1994) Mammalian mitogen-activated protein kinase kinase kinase (MEKK) can function in a yeast mitogen-activated protein kinase pathway downstream of protein kinase C. *Proceedings of the National Academy of Sciences of the United States of America* **91**: 4925–9
- Bossis I, Roden RBS, Gambhira R, Yang R, Tagaya M, Howley PM & Meneses PI (2005) Interaction of tSNARE syntaxin 18 with the papillomavirus minor capsid protein mediates infection. *Journal of virology* **79**: 6723–31
- Bostock C, Prescott DM & Kirkpatrick JB (1971) An evaluation of the double thymidine block for synchronizing mammalian cells at the G1-S border\*1. *Experimental Cell Research* **68**: 163–168
- Boston PF, Jackson P & Thompson RJ (1982) Human 14-3-3 protein: radioimmunoassay, tissue distribution, and cerebrospinal fluid levels in patients with neurological disorders. *Journal of neurochemistry* **38**: 1475–82
- Bousarghin L, Touzé A, Sizaret PY & Coursaget P (2003) Human papillomavirus types 16, 31, and 58 use different endocytosis pathways to enter cells. *Journal of virology* **77**: 3846–50
- Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B & Behr JP (1995) A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proceedings of the National Academy of Sciences of the United States of America* **92**: 7297–301
- Boutros R, Lobjois V & Ducommun B (2007) CDC25 phosphatases in cancer cells: key players? Good targets? *Nature reviews. Cancer* **7**: 495–507
- Bouvard V, Baan R, Straif K, Grosse Y, Secretan B, El Ghissassi F, Benbrahim-Tallaa L, Guha N, Freeman C, Galichet L & Coglianò V (2009) A review of human carcinogens--Part B: biological agents. *The lancet oncology* **10**: 321–2
- Bouwes Bavinck JN, Feltkamp M, Struijk L & ter Schegget J (2001) Human papillomavirus infection and skin cancer risk in organ transplant recipients. *The journal of investigative dermatology. Symposium proceedings / the Society for Investigative Dermatology, Inc. [and] European Society for Dermatological Research* **6**: 207–11
- Bradlow HL, Hershcopf RJ, Martucci CP & Fishman J (1985) Estradiol 16 alpha-hydroxylation in the mouse correlates with mammary tumor

- incidence and presence of murine mammary tumor virus: a possible model for the hormonal etiology of breast cancer in humans. *Proceedings of the National Academy of Sciences of the United States of America* **82**: 6295–9
- Brake T & Lambert PF (2005) Estrogen contributes to the onset, persistence, and malignant progression of cervical cancer in a human papillomavirus-transgenic mouse model. *Proceedings of the National Academy of Sciences of the United States of America* **102**: 2490–5
- Braselmann S & McCormick F (1995) Bcr and Raf form a complex in vivo via 14-3-3 proteins. *The EMBO journal* **14**: 4839–48
- Brehm A, Nielsen SJ, Miska EA, McCance DJ, Reid JL, Bannister AJ & Kouzarides T (1999) The E7 oncoprotein associates with Mi2 and histone deacetylase activity to promote cell growth. *The EMBO journal* **18**: 2449–58
- Brimer N, Lyons C & Vande Pol SB (2007) Association of E6AP (UBE3A) with human papillomavirus type 11 E6 protein. *Virology* **358**: 303–10
- Brimer N & Vande Pol SB (2014) Papillomavirus E6 PDZ interactions can be replaced by repression of p53 to promote episomal human papillomavirus genome maintenance. *Journal of virology* **88**: 3027–30
- Brown DR, Kitchin D, Qadadri B, Neptune N, Batteiger T & Ermel A (2006) The human papillomavirus type 11 E1–E4 protein is a transglutaminase 3 substrate and induces abnormalities of the cornified cell envelope. *Virology* **345**: 290–8
- Brunet A, Kanai F, Stehn J, Xu J, Sarbassova D, Frangioni J V, Dalal SN, DeCaprio JA, Greenberg ME & Yaffe MB (2002) 14-3-3 transits to the nucleus and participates in dynamic nucleocytoplasmic transport. *J Cell Biol* **156**: 817–828
- Bryan JT & Brown DR (2001) Transmission of human papillomavirus type 11 infection by desquamated cornified cells. *Virology* **281**: 35–42
- Bryan JT, Han A, Fife KH & Brown DR (2000) The human papillomavirus type 11 E1E4 protein is phosphorylated in genital epithelium. *Virology* **268**: 430–9
- Bryant PJ & Huwe A (2000) LAP proteins: what's up with epithelia? *Nature cell biology* **2**: E141–3

- Buck CB, Cheng N, Thompson CD, Lowy DR, Steven AC, Schiller JT & Trus BL (2008) Arrangement of L2 within the papillomavirus capsid. *Journal of virology* **82**: 5190–7
- Bugler B, Quaranta M, Aressy B, Brezak M-C, Prevost G & Ducommun B (2006) Genotoxic-activated G2-M checkpoint exit is dependent on CDC25B phosphatase expression. *Molecular cancer therapeutics* **5**: 1446–51
- Bulavin D V, Higashimoto Y, Popoff IJ, Gaarde WA, Basrur V, Potapova O, Appella E & Fornace Jr. AJ (2001) Initiation of a G2/M checkpoint after ultraviolet radiation requires p38 kinase. *Nature* **411**: 102–107
- Burd EM (2003) Human Papillomavirus and Cervical Cancer. *Clinical Microbiology Reviews* **16**: 1–17
- Butz K, Shahabeddin L, Geisen C, Spitkovsky D, Ullmann A & Hoppe-Seyler F (1995) Functional p53 protein in human papillomavirus-positive cancer cells. *Oncogene* **10**: 927–36
- Cahill CM, Tzivion G, Nasrin N, Ogg S, Dore J, Ruvkun G & Alexander-Bridges M (2001) Phosphatidylinositol 3-kinase signaling inhibits DAF-16 DNA binding and function via 14-3-3-dependent and 14-3-3-independent pathways. *The Journal of biological chemistry* **276**: 13402–10
- Cam H & Dynlacht BD (2003) Emerging roles for E2F: beyond the G1/S transition and DNA replication. *Cancer cell* **3**: 311–6
- Campbell JK, Gurung R, Romero S, Speed CJ, Andrews RK, Berndt MC & Mitchell CA (1997) Activation of the 43 kDa inositol polyphosphate 5-phosphatase by 14-3-3zeta. *Biochemistry* **36**: 15363–70
- Celis JE, Gesser B, Rasmussen HH, Madsen P, Leffers H, Dejgaard K, Honore B, Olsen E, Ratz G & Lauridsen JB (1990) Comprehensive two-dimensional gel protein databases offer a global approach to the analysis of human cells: the transformed amnion cells (AMA) master database and its link to genome DNA sequence data. *Electrophoresis* **11**: 989–1071
- Chan PK (2012) Human papillomavirus type 58: the unique role in cervical cancers in East Asia. *Cell & bioscience* **2**: 17
- Chan PM, Ng Y-W & Manser E (2011) A robust protocol to map binding sites of the 14-3-3 interactome: Cdc25C requires phosphorylation of both S216 and S263 to bind 14-3-3. *Molecular & cellular proteomics: MCP* **10**: M110.005157

- Chan WK, Chong T, Bernard HU & Klock G (1990) Transcription of the transforming genes of the oncogenic human papillomavirus-16 is stimulated by tumor promoters through AP1 binding sites. *Nucleic acids research* **18**: 763–9
- Chand V, John R, Jaiswal N, Johar S & Nag A (2014) High Risk HPV16E6 Stimulates hADA3 Degradation by Enhancing its SUMOylation. *Carcinogenesis*
- Chandrasekhar A, Kalmykov EA, Polusani SR, Mathis SA, Zucker SN & Nicholson BJ (2013) Intercellular redistribution of cAMP underlies selective suppression of cancer cell growth by connexin26. *PloS one* **8**: e82335
- Chang Z, Ju H, Ling J, Zhuang Z, Li Z, Wang H, Fleming JB, Freeman JW, Yu D, Huang P & Chiao PJ (2014) Cooperativity of oncogenic K-ras and downregulated p16/INK4A in human pancreatic tumorigenesis. *PloS one* **9**: e101452
- Chaturvedi P, Eng WK, Zhu Y, Mattern MR, Mishra R, Hurle MR, Zhang X, Annan RS, Lu Q, Faucette LF, Scott GF, Li X, Carr SA, Johnson RK, Winkler JD & Zhou BB (1999) Mammalian Chk2 is a downstream effector of the ATM-dependent DNA damage checkpoint pathway. *Oncogene* **18**: 4047–54
- Chaudhri M, Scarabel M & Aitken A (2003) Mammalian and yeast 14-3-3 isoforms form distinct patterns of dimers in vivo. *Biochemical and biophysical research communications* **300**: 679–85
- Chen J & Zhang M (2013) The Par3/Par6/aPKC complex and epithelial cell polarity. *Experimental cell research* **319**: 1357–64
- Chen M-S, Ryan CE & Piwnica-Worms H (2003) Chk1 kinase negatively regulates mitotic function of Cdc25A phosphatase through 14-3-3 binding. *Molecular and cellular biology* **23**: 7488–97
- Cheng M, Olivier P, Diehl JA, Fero M, Roussel MF, Roberts JM & Sherr CJ (1999) The p21(Cip1) and p27(Kip1) CDK “inhibitors” are essential activators of cyclin D-dependent kinases in murine fibroblasts. *The EMBO journal* **18**: 1571–83
- Chiang CW, Harris G, Ellig C, Masters SC, Subramanian R, Shenolikar S, Wadzinski BE & Yang E (2001) Protein phosphatase 2A activates the proapoptotic function of BAD in interleukin-3-dependent lymphoid cells by a mechanism requiring 14-3-3 dissociation. *Blood* **97**: 1289–97

- Choi M, Lee S, Choi T & Lee C (2014) Roles of the PDZ domain-binding motif of the human papillomavirus type 16 E6 on the immortalization and differentiation of primary human foreskin keratinocytes. *Virus genes* **48**: 224–32
- Chong T, Apt D, Gloss B, Isa M & Bernard HU (1991) The enhancer of human papillomavirus type 16: binding sites for the ubiquitous transcription factors oct-1, NFA, TEF-2, NF1, and AP-1 participate in epithelial cell-specific transcription. *Journal of virology* **65**: 5933–43
- Chou S-T, Yen Y-C, Lee C-M & Chen M-S (2010) Pro-apoptotic role of Cdc25A: activation of cyclin B1/Cdc2 by the Cdc25A C-terminal domain. *The Journal of biological chemistry* **285**: 17833–45
- Chow LT & Broker TR (1997) In vitro experimental systems for HPV: Epithelial raft cultures for investigations of viral reproduction and pathogenesis and for genetic analyses of viral proteins and regulatory sequences. *Clinics in Dermatology* **15**: 217–227
- Chow LT, Nasser M, Wolinsky SM & Broker TR (1987a) Human papillomavirus types 6 and 11 mRNAs from genital condylomata acuminata. *Journal of virology* **61**: 2581–8
- Chow LT, Reilly SS, Broker TR & Taichman LB (1987b) Identification and mapping of human papillomavirus type 1 RNA transcripts recovered from plantar warts and infected epithelial cell cultures. *Journal of virology* **61**: 1913–8
- Chung S-H, Franceschi S & Lambert PF (2010) Estrogen and ERalpha: culprits in cervical cancer? *Trends in endocrinology and metabolism: TEM* **21**: 504–11
- Chung S-H & Lambert PF (2009) Prevention and treatment of cervical cancer in mice using estrogen receptor antagonists. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 19467–72
- Chung S-H, Shin MK, Korach KS & Lambert PF (2013) Requirement for stromal estrogen receptor alpha in cervical neoplasia. *Hormones & cancer* **4**: 50–9
- Chung SH, Wiedmeyer K, Shai A, Korach KS & Lambert PF (2008a) Requirement for estrogen receptor alpha in a mouse model for human papillomavirus-associated cervical cancer. *Cancer Res* **68**: 9928–9934

- Chung S-H, Wiedmeyer K, Shai A, Korach KS & Lambert PF (2008b) Requirement for estrogen receptor alpha in a mouse model for human papillomavirus-associated cervical cancer. *Cancer research* **68**: 9928–34
- Clair S St. & Manfredi JJ (2006) The Dual Specificity Phosphatase Cdc25C is a Direct Target for Transcriptional Repression by the Tumor Suppressor p53. *Cell Cycle* **5**: 709–713
- Clark GJ, Drugan JK, Rossman KL, Carpenter JW, Rogers-Graham K, Fu H, Der CJ & Campbell SL (1997) 14-3-3 zeta negatively regulates raf-1 activity by interactions with the Raf-1 cysteine-rich domain. *The Journal of biological chemistry* **272**: 20990–3
- Classon M & Dyson N (2001) p107 and p130: versatile proteins with interesting pockets. *Experimental cell research* **264**: 135–47
- Clemens KE, Brent R, Gyuris J & Münger K (1995) Dimerization of the human papillomavirus E7 oncoprotein in vivo. *Virology* **214**: 289–93
- Cliby WA, Roberts CJ, Cimprich KA, Stringer CM, Lamb JR, Schreiber SL & Friend SH (1998) Overexpression of a kinase-inactive ATR protein causes sensitivity to DNA-damaging agents and defects in cell cycle checkpoints. *The EMBO journal* **17**: 159–69
- Clifford GM, Smith JS, Aguado T & Franceschi S (2003a) Comparison of HPV type distribution in high-grade cervical lesions and cervical cancer: a meta-analysis. *British journal of cancer* **89**: 101–5
- Clifford GM, Smith JS, Plummer M, Muñoz N & Franceschi S (2003b) Human papillomavirus types in invasive cervical cancer worldwide: a meta-analysis. *British journal of cancer* **88**: 63–73
- Coblitz B, Wu M, Shikano S & Li M (2006) C-terminal binding: an expanded repertoire and function of 14-3-3 proteins. *FEBS letters* **580**: 1531–5
- Cohen D, Fernandez D, Lázaro-Diéguez F & Müsch A (2011) The serine/threonine kinase Par1b regulates epithelial lumen polarity via IRSp53-mediated cell-ECM signaling. *The Journal of cell biology* **192**: 525–40
- Conrad M, Bubb VJ & Schlegel R (1993) The human papillomavirus type 6 and 16 E5 proteins are membrane-associated proteins which associate with the 16-kilodalton pore-forming protein. *Journal of virology* **67**: 6170–8
- Craig AL, Burch L, Vojtesek B, Mikutowska J, Thompson A & Hupp TR (1999) Novel phosphorylation sites of human tumour suppressor protein p53 at

- Ser20 and Thr18 that disrupt the binding of mdm2 (mouse double minute 2) protein are modified in human cancers. *The Biochemical journal* **342** ( Pt 1: 133–41
- Crook T, Almond N, Murray A, Stanley M & Crawford L (1989) Constitutive expression of c-myc oncogene confers hormone independence and enhanced growth-factor responsiveness on cells transformed by human papilloma virus type 16. *Proceedings of the National Academy of Sciences of the United States of America* **86**: 5713–7
- Crook T, Tidy JA & Vousden KH (1991) Degradation of p53 can be targeted by HPV E6 sequences distinct from those required for p53 binding and trans-activation. *Cell* **67**: 547–56
- Crusius K, Auvinen E, Steuer B, Gaissert H & Alonso A (1998) The human papillomavirus type 16 E5-protein modulates ligand-dependent activation of the EGF receptor family in the human epithelial cell line HaCaT. *Experimental cell research* **241**: 76–83
- Crusius K, Rodriguez I & Alonso A (2000) The human papillomavirus type 16 E5 protein modulates ERK1/2 and p38 MAP kinase activation by an EGFR-independent process in stressed human keratinocytes. *Virus genes* **20**: 65–9
- Cullen AP, Reid R, Campion M & Lörincz AT (1991) Analysis of the physical state of different human papillomavirus DNAs in intraepithelial and invasive cervical neoplasm. *Journal of virology* **65**: 606–12
- Culp TD & Christensen ND (2004) Kinetics of in vitro adsorption and entry of papillomavirus virions. *Virology* **319**: 152–61
- Cyphert HA, Alonge KM, Ippagunta SM & Hillgartner FB (2014) Glucagon stimulates hepatic FGF21 secretion through a PKA- and EPAC-dependent posttranscriptional mechanism. *PloS one* **9**: e94996
- Dabydeen SA & Meneses PI (2009) The role of NH4Cl and cysteine proteases in Human Papillomavirus type 16 infection. *Virology journal* **6**: 109
- Dalal SN, Yaffe MB & DeCaprio JA (2004) 14-3-3 family members act coordinately to regulate mitotic progression. *Cell Cycle* **3**: 672–677
- Dalvai M, Mondesert O, Bugler B, Manenti S, Ducommun B & Dozier C (2013) Doxorubicin promotes transcriptional upregulation of Cdc25B in cancer cells by releasing Sp1 from the promoter. *Oncogene* **32**: 5123–8

- Danes CG, Wyszomierski SL, Lu J, Neal CL, Yang W & Yu D (2008) 14-3-3 zeta down-regulates p53 in mammary epithelial cells and confers luminal filling. *Cancer research* **68**: 1760–7
- Danova M, Giordano M, Mazzini G & Riccardi A (1990) Expression of p53 protein during the cell cycle measured by flow cytometry in human leukemia. *Leukemia research* **14**: 417–22
- Darzynkiewicz Z, Staiano-Coico L, Kunicka JE, DeLeo AB & Old LJ (1986) p53 content in relation to cell growth and proliferation in murine L1210 leukemia and normal lymphocytes. *Leukemia research* **10**: 1383–9
- Daub H, Olsen J V, Bairlein M, Gnad F, Oppermann FS, Körner R, Greff Z, Kéri G, Stemmann O & Mann M (2008) Kinase-selective enrichment enables quantitative phosphoproteomics of the kinome across the cell cycle. *Molecular cell* **31**: 438–48
- Davies R, Hicks R, Crook T, Morris J & Vousden K (1993) Human papillomavirus type 16 E7 associates with a histone H1 kinase and with p107 through sequences necessary for transformation. *Journal of virology* **67**: 2521–8
- Davies SP, Reddy H, Caivano M & Cohen P (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *The Biochemical journal* **351**: 95–105
- Davis NM, Sokolosky M, Stadelman K, Abrams SL, Libra M, Candido S, Nicoletti F, Polesel J, Maestro R, D'Assoro A, Drobot L, Rakus D, Gizak A, Laidler P, Dulińska-Litewka J, Basecke J, Mijatovic S, Maksimovic-Ivanic D, Montalto G, Cervello M, et al (2014) Deregulation of the EGFR/PI3K/PTEN/Akt/mTORC1 pathway in breast cancer: possibilities for therapeutic intervention. *Oncotarget* **5**: 4603–50
- Davy CE, Jackson DJ, Wang Q, Raj K, Masterson PJ, Fenner NF, Southern S, Cuthill S, Millar JBA & Doorbar J (2002) Identification of a G2 Arrest Domain in the E1 E4 Protein of Human Papillomavirus Type 16. *Journal of Virology* **76**: 9806–9818
- Day PM, Lowy DR & Schiller JT (2003) Papillomaviruses infect cells via a clathrin-dependent pathway. *Virology* **307**: 1–11
- Day PM, Lowy DR & Schiller JT (2008) Heparan sulfate-independent cell binding and infection with furin-precleaved papillomavirus capsids. *Journal of virology* **82**: 12565–8



- Dellinger RW, Karjian PL & Neuteboom STC (2003) NB1011 induces Ser15 phosphorylation of p53 and activates the G2/M checkpoint. *Anti-cancer drugs* **14**: 449–55
- Delury CP, Marsh EK, James CD, Boon SS, Banks L, Knight GL & Roberts S (2013) The Role of Protein Kinase A Regulation of the E6 PDZ-Binding Domain during the Differentiation-Dependent Life Cycle of Human Papillomavirus Type 18. *Journal of virology* **87**: 9463–72
- DeMasi J, Chao MC, Kumar AS & Howley PM (2007) Bovine papillomavirus E7 oncoprotein inhibits anoikis. *Journal of virology* **81**: 9419–25
- DeMasi J, Huh K-W, Nakatani Y, Münger K & Howley PM (2005) Bovine papillomavirus E7 transformation function correlates with cellular p600 protein binding. *Proceedings of the National Academy of Sciences of the United States of America* **102**: 11486–91
- Demers GW, Foster SA, Halbert CL & Galloway DA (1994) Growth arrest by induction of p53 in DNA damaged keratinocytes is bypassed by human papillomavirus 16 E7. *Proceedings of the National Academy of Sciences of the United States of America* **91**: 4382–6
- Dent P, Jelinek T, Morrison DK, Weber MJ & Sturgill TW (1995) Reversal of Raf-1 activation by purified and membrane-associated protein phosphatases. *Science (New York, N.Y.)* **268**: 1902–6
- Dhillon AS, Yip YY, Grindlay GJ, Pakay JL, Dangers M, Hillmann M, Clark W, Pitt A, Mischak H & Kolch W (2009) The C-terminus of Raf-1 acts as a 14-3-3-dependent activation switch. *Cell Signal* **21**: 1645–1651
- Dobrosotskaya I, Guy RK & James GL (1997) MAGI-1, a Membrane-associated Guanylate Kinase with a Unique Arrangement of Protein-Protein Interaction Domains. *Journal of Biological Chemistry* **272**: 31589–31597
- Dolcet X, Llobet D, Pallares J & Matias-Guiu X (2005) NF- $\kappa$ B in development and progression of human cancer. *Virchows Archiv: an international journal of pathology* **446**: 475–82
- Doorbar J (2006) Molecular biology of human papillomavirus infection and cervical cancer. *Clinical science (London, England: 1979)* **110**: 525–41
- Doorbar J, Ely S, Sterling J, McLean C & Crawford L (1991) Specific interaction between HPV-16 E1-E4 and cytokeratins results in collapse of the epithelial cell intermediate filament network. *Nature* **352**: 824–7

- Doorbar J, Foo C, Coleman N, Medcalf L, Hartley O, Prospero T, Napthine S, Sterling J, Winter G & Griffin H (1997) Characterization of events during the late stages of HPV16 infection in vivo using high-affinity synthetic Fabs to E4. *Virology* **238**: 40–52
- Doorbar J, Parton A, Hartley K, Banks L, Crook T, Stanley M & Crawford L (1990) Detection of novel splicing patterns in a HPV16-containing keratinocyte cell line. *Virology* **178**: 254–62
- Downward J (2003) Targeting RAS signalling pathways in cancer therapy. *Nature reviews. Cancer* **3**: 11–22
- Du X, Fox JE & Pei S (1996) Identification of a binding sequence for the 14-3-3 protein within the cytoplasmic domain of the adhesion receptor, platelet glycoprotein Ib alpha. *The Journal of biological chemistry* **271**: 7362–7
- Dubois T, Howell S, Amess B, Kerai P, Learmonth M, Madrazo J, Chaudhri M, Rittinger K, Scarabel M, Soneji Y & Aitken A (1997a) Structure and sites of phosphorylation of 14-3-3 protein: role in coordinating signal transduction pathways. *Journal of protein chemistry* **16**: 513–22
- Dubois T, Rommel C, Howell S, Steinhussen U, Soneji Y, Morrice N, Moelling K & Aitken A (1997b) 14-3-3 is phosphorylated by casein kinase I on residue 233. Phosphorylation at this site in vivo regulates Raf/14-3-3 interaction. *The Journal of biological chemistry* **272**: 28882–8
- Duensing A, Liu Y, Tseng M, Malumbres M, Barbacid M & Duensing S (2006) Cyclin-dependent kinase 2 is dispensable for normal centrosome duplication but required for oncogene-induced centrosome overduplication. *Oncogene* **25**: 2943–9
- Duensing S, Lee LY, Duensing A, Basile J, Piboonniyom S, Gonzalez S, Crum CP & Munger K (2000) The human papillomavirus type 16 E6 and E7 oncoproteins cooperate to induce mitotic defects and genomic instability by uncoupling centrosome duplication from the cell division cycle. *Proc Natl Acad Sci U S A* **97**: 10002–10007
- Dumaz N & Marais R (2003) Protein kinase A blocks Raf-1 activity by stimulating 14-3-3 binding and blocking Raf-1 interaction with Ras. *The Journal of biological chemistry* **278**: 29819–23
- Dunphy WG & Kumagai A (1991) The cdc25 protein contains an intrinsic phosphatase activity. *Cell* **67**: 189–196
- Durocher D & Jackson SP (2002) The FHA domain. *FEBS letters* **513**: 58–66

- Dürst M, Dzarlieva-Petrusevska RT, Boukamp P, Fusenig NE & Gissmann L (1987) Molecular and cytogenetic analysis of immortalized human primary keratinocytes obtained after transfection with human papillomavirus type 16 DNA. *Oncogene* **1**: 251–6
- Dyson N (1998) The regulation of E2F by pRB-family proteins. *Genes & Development* **12**: 2245–2262
- Dyson N, Guida P, Münger K & Harlow E (1992) Homologous sequences in adenovirus E1A and human papillomavirus E7 proteins mediate interaction with the same set of cellular proteins. *Journal of virology* **66**: 6893–902
- Dyson N, Howley PM, Münger K & Harlow E (1989) The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science (New York, N.Y.)* **243**: 934–7
- Egawa K (2003) Do human papillomaviruses target epidermal stem cells? *Dermatology (Basel, Switzerland)* **207**: 251–4
- Ekholm D, Belfrage P, Manganiello V & Degerman E (1997) Protein kinase A-dependent activation of PDE4 (cAMP-specific cyclic nucleotide phosphodiesterase) in cultured bovine vascular smooth muscle cells. *Biochimica et biophysica acta* **1356**: 64–70
- Ekholm S V & Reed SI (2000) Regulation of G1 cyclin-dependent kinases in the mammalian cell cycle. *Current Opinion in Cell Biology* **12**: 676–684
- Elia AEH, Cantley LC & Yaffe MB (2003) Proteomic screen finds pSer/pThr-binding domain localizing Plk1 to mitotic substrates. *Science (New York, N.Y.)* **299**: 1228–31
- Ellis JJ, Valencia TG, Zeng H, Roberts LD, Deaton RA & Grant SR (2003) CaM kinase II $\delta$  phosphorylation of 14-3-3 $\beta$  in vascular smooth muscle cells: activation of class II HDAC repression. *Molecular and cellular biochemistry* **242**: 153–61
- Elston RC, Napthine S & Doorbar J (1998) The identification of a conserved binding motif within human papillomavirus type 16 E6 binding peptides, E6AP and E6BP. *The Journal of general virology* **79** ( Pt 2): 371–4
- El-Yazbi AF, Cho WJ, Schulz R & Daniel EE (2006) Caveolin-1 knockout alters beta-adrenoceptors function in mouse small intestine. *American journal of physiology. Gastrointestinal and liver physiology* **291**: G1020–30

- Enemark EJ & Joshua-Tor L (2006) Mechanism of DNA translocation in a replicative hexameric helicase. *Nature* **442**: 270–5
- Esmenjaud-Mailhat C, Lobjois V, Froment C, Golsteyn RM, Monsarrat B & Ducommun B (2007) Phosphorylation of CDC25C at S263 controls its intracellular localisation. *FEBS letters* **581**: 3979–85
- Evander M, Frazer IH, Payne E, Qi YM, Hengst K & McMillan NA (1997) Identification of the alpha6 integrin as a candidate receptor for papillomaviruses. *Journal of virology* **71**: 2449–56
- Fan T, Li R, Todd NW, Qiu Q, Fang HB, Wang H, Shen J, Zhao RY, Caraway NP, Katz RL, Stass SA & Jiang F (2007) Up-regulation of 14-3-3zeta in lung cancer and its implication as prognostic and therapeutic target. *Cancer Res* **67**: 7901–7906
- Fanger GR, Widmann C, Porter AC, Sather S, Johnson GL & Vaillancourt RR (1998) 14-3-3 proteins interact with specific MEK kinases. *The Journal of biological chemistry* **273**: 3476–83
- Fanning AS & Anderson JM (1999) PDZ domains: fundamental building blocks in the organization of protein complexes at the plasma membrane. *The Journal of clinical investigation* **103**: 767–72
- Favre M, Breitburd F, Croissant O & Orth G (1977) Chromatin-like structures obtained after alkaline disruption of bovine and human papillomaviruses. *Journal of virology* **21**: 1205–9
- Fehrman F, Klumpp DJ & Laimins LA (2003) Human papillomavirus type 31 E5 protein supports cell cycle progression and activates late viral functions upon epithelial differentiation. *Journal of virology* **77**: 2819–31
- Fehrman F & Laimins LA (2003) Human papillomaviruses: targeting differentiating epithelial cells for malignant transformation. *Oncogene* **22**: 5201–7
- Feng J, Funk W, Wang S, Weinrich S, Avilion A, Chiu C, Adams R, Chang E, Allsopp R, Yu J & al. e. (1995) The RNA component of human telomerase. *Science* **269**: 1236–1241
- Ferguson AT, Evron E, Umbricht CB, Pandita TK, Chan TA, Hermeking H, Marks JR, Lambers AR, Futreal PA, Stampfer MR & Sukumar S (2000) High frequency of hypermethylation at the 14-3-3 sigma locus leads to gene silencing in breast cancer. *Proceedings of the National Academy of Sciences of the United States of America* **97**: 6049–54

- Ferlay J, Shin H-R, Bray F, Forman D, Mathers C & Parkin DM (2010) Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *International journal of cancer. Journal international du cancer* **127**: 2893–917
- Ferlay J, Soerjornataram I, Ervik M, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin D, Forman D & Brad F (2013) GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 Lyon, France
- Fichorova RN (1999) Differential Expression of Immunobiological Mediators by Immortalized Human Cervical and Vaginal Epithelial Cells. *Biology of Reproduction* **60**: 508–514
- Filippa N, Sable CL, Filloux C, Hemmings B & Van Obberghen E (1999) Mechanism of Protein Kinase B Activation by Cyclic AMP-Dependent Protein Kinase. *Mol. Cell. Biol.* **19**: 4989–5000
- Filippova M, Johnson MM, Bautista M, Filippov V, Fodor N, Tungteakkhun SS, Williams K & Duerksen-Hughes PJ (2007) The large and small isoforms of human papillomavirus type 16 E6 bind to and differentially affect procaspase 8 stability and activity. *J Virol* **81**: 4116–4129
- Filippova M, Parkhurst L & Duerksen-Hughes PJ (2004) The human papillomavirus 16 E6 protein binds to Fas-associated death domain and protects cells from Fas-triggered apoptosis. *J Biol Chem* **279**: 25729–25744
- Filippova M, Song H, Connolly JL, Dermody TS & Duerksen-Hughes PJ (2002) The human papillomavirus 16 E6 protein binds to tumor necrosis factor (TNF) R1 and protects cells from TNF-induced apoptosis. *J Biol Chem* **277**: 21730–21739
- Firzlaff JM, Galloway DA, Eisenman RN & Lüscher B (1989) The E7 protein of human papillomavirus type 16 is phosphorylated by casein kinase II. *The New biologist* **1**: 44–53
- Fischer A, Baljuls A, Reinders J, Nekhoroshkova E, Sibilski C, Metz R, Albert S, Rajalingam K, Hekman M & Rapp UR (2009) Regulation of RAF activity by 14-3-3 proteins: RAF kinases associate functionally with both homo- and heterodimeric forms of 14-3-3 proteins. *The Journal of biological chemistry* **284**: 3183–94
- Florin L, Becker KA, Sapp C, Lambert C, Sirma H, Müller M, Streeck RE & Sapp M (2004) Nuclear translocation of papillomavirus minor capsid protein L2 requires Hsc70. *Journal of virology* **78**: 5546–53

- Florin L, Sapp C, Streeck RE & Sapp M (2002) Assembly and translocation of papillomavirus capsid proteins. *Journal of virology* **76**: 10009–14
- Forester CM, Maddox J, Louis J V, Goris J & Virshup DM (2007) Control of mitotic exit by PP2A regulation of Cdc25C and Cdk1. *Proceedings of the National Academy of Sciences of the United States of America* **104**: 19867–72
- Forrest A & Gabrielli B (2001) Cdc25B activity is regulated by 14-3-3. *Oncogene* **20**: 4393–401
- Foster SA, Demers GW, Etscheid BG & Galloway DA (1994) The ability of human papillomavirus E6 proteins to target p53 for degradation in vivo correlates with their ability to abrogate actinomycin D-induced growth arrest. *Journal of virology* **68**: 5698–705
- Fouts ET, Yu X, Egelman EH & Botchan MR (1999) Biochemical and electron microscopic image analysis of the hexameric E1 helicase. *The Journal of biological chemistry* **274**: 4447–58
- Franks DJ, Whitfield JF & Durkin JP (1987) Viral p21 Ki-RAS protein: a potent intracellular mitogen that stimulates adenylate cyclase activity in early G1 phase of cultured rat cells. *Journal of cellular biochemistry* **33**: 87–94
- Frattoni MG, Lim HB & Laimins LA (1996) In vitro synthesis of oncogenic human papillomaviruses requires episomal genomes for differentiation-dependent late expression. *Proceedings of the National Academy of Sciences of the United States of America* **93**: 3062–7
- Freed E, Symons M, Macdonald S, McCormick F & Ruggieri R (1994) Binding of 14-3-3 proteins to the protein kinase Raf and effects on its activation. *Science* **265**: 1713–1716
- Frisch SM & Screaton RA (2001) Anoikis mechanisms. *Current Opinion in Cell Biology* **13**: 555–562
- Fu H, Coburn J & Collier RJ (1993) The eukaryotic host factor that activates exoenzyme S of *Pseudomonas aeruginosa* is a member of the 14-3-3 protein family. *Proceedings of the National Academy of Sciences of the United States of America* **90**: 2320–4
- Fu H, Subramanian RR & Masters SC (2000) 14-3-3 proteins: structure, function, and regulation. *Annual review of pharmacology and toxicology* **40**: 617–47

- Fu H, Xia K, Pallas D, Cui C, Conroy K, Narsimhan R, Mamon H, Collier R & Roberts T (1994) Interaction of the protein kinase Raf-1 with 14-3-3 proteins. *Science* **266**: 126–129
- Fujita N, Sato S, Katayama K & Tsuruo T (2002) Akt-dependent phosphorylation of p27Kip1 promotes binding to 14-3-3 and cytoplasmic localization. *The Journal of biological chemistry* **277**: 28706–13
- Funk JO, Waga S, Harry JB, Espling E, Stillman B & Galloway DA (1997) Inhibition of CDK activity and PCNA-dependent DNA replication by p21 is blocked by interaction with the HPV-16 E7 oncoprotein. *Genes & Development* **11**: 2090–2100
- Galabova-Kovacs G, Kolbus A, Matzen D, Meissl K, Piazzolla D, Rubiolo C, Steinitz K & Baccarini M (2006) ERK and beyond: insights from B-Raf and Raf-1 conditional knockouts. *Cell cycle (Georgetown, Tex.)* **5**: 1514–8
- Gao YL, Twiggs LB, Leung BS, Yu WC, Potish RA, Okagaki T, Adcock LL & Prem KA (1983) Cytoplasmic estrogen and progesterone receptors in primary cervical carcinoma: clinical and histopathologic correlates. *American journal of obstetrics and gynecology* **146**: 299–306
- Gao Z, Chen T, Weber MJ & Linden J (1999) A2B Adenosine and P2Y2 Receptors Stimulate Mitogen-activated Protein Kinase in Human Embryonic Kidney-293 Cells: CROSS-TALK BETWEEN CYCLIC AMP AND PROTEIN KINASE C PATHWAYS. *Journal of Biological Chemistry* **274**: 5972–5980
- Garcia-Guzman M, Dolfi F, Russello M & Vuori K (1999) Cell Adhesion Regulates the Interaction between the Docking Protein p130Cas and the 14-3-3 Proteins. *J. Biol. Chem.* **274**: 5762–5768
- Gardiol D, Kühne C, Glaunsinger B, Lee SS, Javier R & Banks L (1999) Oncogenic human papillomavirus E6 proteins target the discs large tumour suppressor for proteasome-mediated degradation. *Oncogene* **18**: 5487–96
- Gardoni F, Marcello E & Di Luca M (2009) Postsynaptic density-membrane associated guanylate kinase proteins (PSD-MAGUKs) and their role in CNS disorders. *Neuroscience* **158**: 324–33
- Garner-Hamrick PA, Fostel JM, Chien W-M, Banerjee NS, Chow LT, Broker TR & Fisher C (2004) Global effects of human papillomavirus type 18 E6/E7 in an organotypic keratinocyte culture system. *Journal of virology* **78**: 9041–50

- Garnett MJ, Rana S, Paterson H, Barford D & Marais R (2005) Wild-type and mutant B-RAF activate C-RAF through distinct mechanisms involving heterodimerization. *Molecular cell* **20**: 963–9
- Gateff E & Schneiderman HA (1974) Developmental capacities of benign and malignant neoplasms of *Drosophila*. *Wilhelm Roux Archiv für Entwicklungsmechanik der Organismen* **176**: 23–65
- Gaudet S, Branton D & Lue RA (2000) Characterization of PDZ-binding kinase, a mitotic kinase. *Proceedings of the National Academy of Sciences of the United States of America* **97**: 5167–72
- Gautier J, Solomon MJ, Booher RN, Bazan JF & Kirschner MW (1991) cdc25 is a specific tyrosine phosphatase that directly activates p34cdc2. *Cell* **67**: 197–211
- De Geest K, Turyk ME, Hosken MI, Hudson JB, Laimins LA & Wilbanks GD (1993) Growth and differentiation of human papillomavirus type 31b positive human cervical cell lines. *Gynecologic oncology* **49**: 303–10
- Gewin L, Myers H, Kiyono T & Galloway DA (2004) Identification of a novel telomerase repressor that interacts with the human papillomavirus type-16 E6/E6-AP complex. *Genes & development* **18**: 2269–82
- Ghadimi BM, Grade M, Liersch T, Langer C, Siemer A, Füzesi L & Becker H (2003) Gain of chromosome 8q23-24 is a predictive marker for lymph node positivity in colorectal cancer. *Clinical cancer research: an official journal of the American Association for Cancer Research* **9**: 1808–14
- Giarre M, Caldeira S, Malanchi I, Ciccolini F, Leão MJ & Tommasino M (2001) Induction of pRb degradation by the human papillomavirus type 16 E7 protein is essential to efficiently overcome p16INK4a-imposed G1 cell cycle Arrest. *Journal of virology* **75**: 4705–12
- Giles N, Forrest A & Gabrielli B (2003) 14-3-3 acts as an intramolecular bridge to regulate cdc25B localization and activity. *J Biol Chem* **278**: 28580–28587
- Gillison ML & Shah K V. (2003) Chapter 9: Role of Mucosal Human Papillomavirus in Nongenital Cancers. *JNCI Monographs* **2003**: 57–65
- Giroglou T, Florin L, Schäfer F, Streeck RE & Sapp M (2001) Human papillomavirus infection requires cell surface heparan sulfate. *Journal of virology* **75**: 1565–70



- Glaunsinger BA, Lee SS, Thomas M, Banks L & Javier R (2000) Interactions of the PDZ-protein MAGI-1 with adenovirus E4-ORF1 and high-risk papillomavirus E6 oncoproteins. *Oncogene* **19**: 5270–80
- Goldfarb DS, Corbett AH, Mason DA, Harreman MT & Adam SA (2004) Importin alpha: a multipurpose nuclear-transport receptor. *Trends in cell biology* **14**: 505–14
- Gonzalez SL, Stremlau M, He X, Basile JR & Münger K (2001) Degradation of the retinoblastoma tumor suppressor by the human papillomavirus type 16 E7 oncoprotein is important for functional inactivation and is separable from proteasomal degradation of E7. *Journal of virology* **75**: 7583–91
- González-Mariscal L, Betanzos A & Avila-Flores A (2000) MAGUK proteins: structure and role in the tight junction. *Seminars in cell & developmental biology* **11**: 315–24
- Goodwin EC & DiMaio D (2000) Repression of human papillomavirus oncogenes in HeLa cervical carcinoma cells causes the orderly reactivation of dormant tumor suppressor pathways. *Proc Natl Acad Sci U S A* **97**: 12513–12518
- Graham S V (2010) Human papillomavirus: gene expression, regulation and prospects for novel diagnostic methods and antiviral therapies. *Future microbiology* **5**: 1493–506
- Grayson W, Taylor LF, Allard U, Tiltman AJ & Rhemtula HA (2002) Detection of human papillomavirus in large cell neuroendocrine carcinoma of the uterine cervix: a study of 12 cases. *Journal of Clinical Pathology* **55**: 108–114
- Green KA & Streuli CH (2004) Apoptosis regulation in the mammary gland. *Cellular and molecular life sciences : CMLS* **61**: 1867–83
- Greenfield I, Nickerson J, Penman S & Stanley M (1991) Human papillomavirus 16 E7 protein is associated with the nuclear matrix. *Proceedings of the National Academy of Sciences of the United States of America* **88**: 11217–21
- Greider CW & Blackburn EH (1985) Identification of a specific telomere terminal transferase activity in tetrahymena extracts. *Cell* **43**: 405–413
- Grossman SR, Mora R & Laimins LA (1989) Intracellular localization and DNA-binding properties of human papillomavirus type 18 E6 protein expressed with a baculovirus vector. *Journal of virology* **63**: 366–74

- Grozinger CM & Schreiber SL (2000) Regulation of histone deacetylase 4 and 5 and transcriptional activity by 14-3-3-dependent cellular localization. *Proceedings of the National Academy of Sciences of the United States of America* **97**: 7835–40
- Gu Y-M, Jin Y-H, Choi J-K, Baek K-H, Yeo C-Y & Lee K-Y (2006) Protein kinase A phosphorylates and regulates dimerization of 14-3-3 $\zeta$
- Guccione E, Massimi P, Bernat A & Banks L (2002) Comparative analysis of the intracellular location of the high- and low-risk human papillomavirus oncoproteins. *Virology* **293**: 20–5
- Gudas JM, Oka M, Diella F, Trepel J & Cowan KH (1994) Expression of wild-type p53 during the cell cycle in normal human mammary epithelial cells. *Cell growth & differentiation: the molecular biology journal of the American Association for Cancer Research* **5**: 295–304
- Hagensee ME, Yaegashi N & Galloway DA (1993) Self-assembly of human papillomavirus type 1 capsids by expression of the L1 protein alone or by coexpression of the L1 and L2 capsid proteins. *Journal of virology* **67**: 315–22
- Halazonetis TD, Davis LJ & Kandil AN (1993) Wild-type p53 adopts a “mutant”-like conformation when bound to DNA. *The EMBO journal* **12**: 1021–8
- Hammet A, Pike BL, McNees CJ, Conlan LA, Tennis N & Heierhorst J (2003) FHA domains as phospho-threonine binding modules in cell signaling. *IUBMB life* **55**: 23–7
- Hanahan D & Weinberg RA (2000) The Hallmarks of Cancer. *Cell* **100**: 57–70
- Harper JW, Elledge SJ, Keyomarsi K, Dynlacht B, Tsai LH, Zhang P, Dobrowolski S, Bai C, Connell-Crowley L & Swindell E (1995) Inhibition of cyclin-dependent kinases by p21. *Molecular biology of the cell* **6**: 387–400
- Harwood CA, McGregor JM, Proby CM & Breuer J (1999) Human papillomavirus and the development of non-melanoma skin cancer. *Journal of clinical pathology* **52**: 249–53
- Hawley-Nelson P, Vousden KH, Hubbert NL, Lowy DR & Schiller JT (1989) HPV16 E6 and E7 proteins cooperate to immortalize human foreskin keratinocytes. *Embo J* **8**: 3905–3910

- Hayden MS & Ghosh S (2004) Signaling to NF-kappaB. *Genes & development* **18**: 2195–224
- He W, Staples D, Smith C & Fisher C (2003) Direct activation of cyclin-dependent kinase 2 by human papillomavirus E7. *Journal of virology* **77**: 10566–74
- Helt AM & Galloway DA (2001) Destabilization of the retinoblastoma tumor suppressor by human papillomavirus type 16 E7 is not sufficient to overcome cell cycle arrest in human keratinocytes. *Journal of virology* **75**: 6737–47
- Hengstermann A, D'silva MA, Kuballa P, Butz K, Hoppe-Seyler F & Scheffner M (2005) Growth suppression induced by downregulation of E6-AP expression in human papillomavirus-positive cancer cell lines depends on p53. *Journal of virology* **79**: 9296–300
- Herber R, Liem A, Pitot H & Lambert PF (1996) Squamous epithelial hyperplasia and carcinoma in mice transgenic for the human papillomavirus type 16 E7 oncogene. *Journal of virology* **70**: 1873–81
- Herfs M, Yamamoto Y, Laury A, Wang X, Nucci MR, McLaughlin-Drubin ME, Mürger K, Feldman S, McKeon FD, Xian W & Crum CP (2012) A discrete population of squamocolumnar junction cells implicated in the pathogenesis of cervical cancer. *Proceedings of the National Academy of Sciences of the United States of America* **109**: 10516–21
- Hermeking H (2003) The 14-3-3 cancer connection. *Nature Reviews Cancer* **3**: 931–943
- Hermeking H & Benzinger A (2006) 14-3-3 proteins in cell cycle regulation. *Semin Cancer Biol* **16**: 183–192
- Hermeking H, Lengauer C, Polyak K, He TC, Zhang L, Thiagalingam S, Kinzler KW & Vogelstein B (1997) 14-3-3 sigma is a p53-regulated inhibitor of G2/M progression. *Mol Cell* **1**: 3–11
- Higareda-Almaraz JC, Valtierra-Gutiérrez IA, Hernandez-Ortiz M, Contreras S, Hernandez E, Encarnación-Guevara S & Encarnacion S (2013) Analysis and prediction of pathways in HeLa cells by integrating biological levels of organization with systems-biology approaches. *PloS one* **8**: e65433
- Hodgkinson VC, Agarwal V, ELFadl D, Fox JN, McManus PL, Mahapatra TK, Kneeshaw PJ, Drew PJ, Lind MJ & Cawkwell L (2012a) Pilot and feasibility study: comparative proteomic analysis by 2-DE MALDI TOF/TOF MS reveals 14-3-3 proteins as putative biomarkers of response

- to neoadjuvant chemotherapy in ER-positive breast cancer. *Journal of proteomics* **75**: 2745–52
- Hodgkinson VC, ELFadl D, Agarwal V, Garimella V, Russell C, Long ED, Fox JN, McManus PL, Mahapatra TK, Kneeshaw PJ, Drew PJ, Lind MJ & Cawkwell L (2012b) Proteomic identification of predictive biomarkers of resistance to neoadjuvant chemotherapy in luminal breast cancer: a possible role for 14-3-3 theta/tau and tBID? *Journal of proteomics* **75**: 1276–83
- Hoffmann I, Clarke PR, Marcote MJ, Karsenti E & Draetta G (1993) Phosphorylation and activation of human cdc25-C by cdc2--cyclin B and its involvement in the self-amplification of MPF at mitosis. *The EMBO journal* **12**: 53–63
- Holm R, Ali T, Svendsrud DH, Nesland JM, Kristensen GB & Lyng H (2009) Expression of 14-3-3sigma in cervical squamous cell carcinomas: relationship with clinical outcome. *Oncology reports* **22**: 11–5
- Hong H-Y, Jeon W-K, Bae E-J, Kim S-T, Lee H-J, Kim S-J & Kim B-C (2010) 14-3-3 sigma and 14-3-3 zeta plays an opposite role in cell growth inhibition mediated by transforming growth factor-beta 1. *Molecules and cells* **29**: 305–9
- Hoover AC, Strand GL, Nowicki PN, Anderson ME, Vermeer PD, Klingelutz AJ, Bossler AD, Pottala J V, Hendriks WJAJ & Lee JH (2009) Impaired PTPN13 phosphatase activity in spontaneous or HPV-induced squamous cell carcinomas potentiates oncogene signaling through the MAP kinase pathway. *Oncogene* **28**: 3960–70
- Howie HL, Koop JJ, Weese J, Robinson K, Wipf G, Kim L & Galloway DA (2011) Beta-HPV 5 and 8 E6 promote p300 degradation by blocking AKT/p300 association. *PLoS pathogens* **7**: e1002211
- Huang X-Y, Ke A-W, Shi G-M, Zhang X, Zhang C, Shi Y-H, Wang X-Y, Ding Z-B, Xiao Y-S, Yan J, Qiu S-J, Fan J & Zhou J (2013)  $\alpha$ B-crystallin complexes with 14-3-3 $\zeta$  to induce epithelial-mesenchymal transition and resistance to sorafenib in hepatocellular carcinoma. *Hepatology (Baltimore, Md.)* **57**: 2235–47
- Huh K, Zhou X, Hayakawa H, Cho J-Y, Libermann TA, Jin J, Harper JW & Munger K (2007) Human papillomavirus type 16 E7 oncoprotein associates with the cullin 2 ubiquitin ligase complex, which contributes to degradation of the retinoblastoma tumor suppressor. *Journal of virology* **81**: 9737–47

- Huh K-W, DeMasi J, Ogawa H, Nakatani Y, Howley PM & Münger K (2005) Association of the human papillomavirus type 16 E7 oncoprotein with the 600-kDa retinoblastoma protein-associated factor, p600. *Proceedings of the National Academy of Sciences of the United States of America* **102**: 11492–7
- Huibregtse JM, Scheffner M & Howley PM (1991) A cellular protein mediates association of p53 with the E6 oncoprotein of human papillomavirus types 16 or 18. *The EMBO journal* **10**: 4129–35
- Huibregtse JM, Scheffner M & Howley PM (1993a) Cloning and expression of the cDNA for E6-AP, a protein that mediates the interaction of the human papillomavirus E6 oncoprotein with p53. *Molecular and cellular biology* **13**: 775–84
- Huibregtse JM, Scheffner M & Howley PM (1993b) Localization of the E6-AP regions that direct human papillomavirus E6 binding, association with p53, and ubiquitination of associated proteins. *Molecular and cellular biology* **13**: 4918–27
- Humbert PO, Grzeschik NA, Brumby AM, Galea R, Elsum I & Richardson HE (2008) Control of tumourigenesis by the Scribble/Dlg/Lgl polarity module. *Oncogene* **27**: 6888–907
- Hummel M, Hudson JB & Laimins LA (1992) Differentiation-induced and constitutive transcription of human papillomavirus type 31b in cell lines containing viral episomes. *Journal of virology* **66**: 6070–80
- Hupp TR, Meek DW, Midgley CA & Lane DP (1992) Regulation of the specific DNA binding function of p53. *Cell* **71**: 875–886
- Hurlin PJ, Kaur P, Smith PP, Perez-Reyes N, Blanton RA & McDougall JK (1991) Progression of human papillomavirus type 18-immortalized human keratinocytes to a malignant phenotype. *Proceedings of the National Academy of Sciences of the United States of America* **88**: 570–4
- IARC Working Group on the Evaluation of Carcinogenic Risks to Humans (2007) Human papillomaviruses. *IARC monographs on the evaluation of carcinogenic risks to humans / World Health Organization, International Agency for Research on Cancer* **90**: 1–636
- Imai Y, Matsushima Y, Sugimura T & Terada M (1991) Purification and characterization of human papillomavirus type 16 E7 protein with preferential binding capacity to the underphosphorylated form of retinoblastoma gene product. *Journal of virology* **65**: 4966–72

- Imai Y, Tsunokawa Y, Sugimura T & Terada M (1989) Purification and DNA-binding properties of human papillomavirus type 16 E6 protein expressed in *Escherichia coli*. *Biochem Biophys Res Commun* **164**: 1402–1410
- Ishiwatari H, Hayasaka N, Inoue H, Yutsudo M & Hakura A (1994) Degradation of p53 only is not sufficient for the growth stimulatory effect of human papillomavirus 16 E6 oncoprotein in human embryonic fibroblasts. *Journal of medical virology* **44**: 243–9
- Islas S, Vega J, Ponce L & González-Mariscal L (2002) Nuclear localization of the tight junction protein ZO-2 in epithelial cells. *Experimental cell research* **274**: 138–48
- Iwata N, Yamamoto H, Sasaki S, Itoh F, Suzuki H, Kikuchi T, Kaneto H, Iku S, Ozeki I, Karino Y, Satoh T, Toyota J, Satoh M, Endo T & Imai K (2000) Frequent hypermethylation of CpG islands and loss of expression of the 14-3-3 sigma gene in human hepatocellular carcinoma. *Oncogene* **19**: 5298–302
- Jackson S, Harwood C, Thomas M, Banks L & Storey A (2000) Role of Bak in UV-induced apoptosis in skin cancer and abrogation by HPV E6 proteins. *Genes & development* **14**: 3065–73
- Jackson S & Storey A (2000) E6 proteins from diverse cutaneous HPV types inhibit apoptosis in response to UV damage. *Oncogene* **19**: 592–8
- Jacob L, Oppen M, Metzroth B, Phannavong B & Mechler BM (1987) Structure of the I(2)gl gene of *Drosophila* and delimitation of its tumor suppressor domain. *Cell* **50**: 215–225
- James A, Wang Y, Raje H, Rosby R & DiMario P (2014) Nucleolar stress with and without p53. *Nucleus (Austin, Tex.)* **5**:
- James MA, Lee JH & Klingelutz AJ (2006) HPV16-E6 associated hTERT promoter acetylation is E6AP dependent, increased in later passage cells and enhanced by loss of p300. *International journal of cancer. Journal international du cancer* **119**: 1878–85
- Jancík S, Drábek J, Radzioch D & Hajdúch M (2010) Clinical relevance of KRAS in human cancers. *Journal of biomedicine & biotechnology* **2010**: 150960
- Jang JS, Cho HY, Lee YJ, Ha WS & Kim HW (2004) The differential proteome profile of stomach cancer: identification of the biomarker candidates. *Oncol Res* **14**: 491–499

- Jang MK, Shen K & McBride AA (2014) Papillomavirus genomes associate with BRD4 to replicate at fragile sites in the host genome. *PLoS pathogens* **10**: e1004117
- Jaramillo BE, Ponce A, Moreno J, Betanzos A, Huerta M, Lopez-Bayghen E & Gonzalez-Mariscal L (2004) Characterization of the tight junction protein ZO-2 localized at the nucleus of epithelial cells. *Experimental cell research* **297**: 247–58
- Javier RT (2008) Cell polarity proteins: common targets for tumorigenic human viruses. *Oncogene* **27**: 7031–46
- Jenkins D (2007) Histopathology and cytopathology of cervical cancer. *Disease markers* **23**: 199–212
- Jeon S, Allen-Hoffmann BL & Lambert PF (1995) Integration of human papillomavirus type 16 into the human genome correlates with a selective growth advantage of cells. *Journal of virology* **69**: 2989–97
- Jeon S & Lambert PF (1995) Integration of human papillomavirus type 16 DNA into the human genome leads to increased stability of E6 and E7 mRNAs: implications for cervical carcinogenesis. *Proceedings of the National Academy of Sciences of the United States of America* **92**: 1654–8
- Jha S, Vande Pol S, Banerjee NS, Dutta AB, Chow LT & Dutta A (2010) Destabilization of TIP60 by human papillomavirus E6 results in attenuation of TIP60-dependent transcriptional regulation and apoptotic pathway. *Molecular cell* **38**: 700–11
- Jin J, Shirogane T, Xu L, Nalepa G, Qin J, Elledge SJ & Harper JW (2003) SCFbeta-TRCP links Chk1 signaling to degradation of the Cdc25A protein phosphatase. *Genes & development* **17**: 3062–74
- Jing M, Bohl J, Brimer N, Kinter M & Vande Pol SB (2007) Degradation of tyrosine phosphatase PTPN3 (PTPH1) by association with oncogenic human papillomavirus E6 proteins. *Journal of virology* **81**: 2231–9
- Jones DH, Ley S & Aitken A (1995a) Isoforms of 14-3-3 protein can form homo- and heterodimers in vivo and in vitro: implications for function as adapter proteins. *FEBS Letters* **368**: 55–58
- Jones DH, Martin H, Madrazo J, Robinson KA, Nielsen P, Roseboom PH, Patel Y, Howell SA & Aitken A (1995b) Expression and structural analysis of 14-3-3 proteins. *Journal of molecular biology* **245**: 375–84

- Jones DL, Alani RM & Munger K (1997a) The human papillomavirus E7 oncoprotein can uncouple cellular differentiation and proliferation in human keratinocytes by abrogating p21Cip1-mediated inhibition of cdk2. *Genes Dev* **11**: 2101–2111
- Jones DL, Alani RM & Munger K (1997b) The human papillomavirus E7 oncoprotein can uncouple cellular differentiation and proliferation in human keratinocytes by abrogating p21Cip1-mediated inhibition of cdk2. *Genes & Development* **11**: 2101–2111
- Joyce JG, Tung J-S, Przysiecki CT, Cook JC, Lehman ED, Sands JA, Jansen KU & Keller PM (1999) The L1 Major Capsid Protein of Human Papillomavirus Type 11 Recombinant Virus-like Particles Interacts with Heparin and Cell-surface Glycosaminoglycans on Human Keratinocytes. *J. Biol. Chem.* **274**: 5810–5822
- Kämper N, Day PM, Nowak T, Selinka H-C, Florin L, Bolscher J, Hilbig L, Schiller JT & Sapp M (2006) A membrane-destabilizing peptide in capsid protein L2 is required for egress of papillomavirus genomes from endosomes. *Journal of virology* **80**: 759–68
- Kanai F, Marignani PA, Sarbassova D, Yagi R, Hall RA, Donowitz M, Hisaminato A, Fujiwara T, Ito Y, Cantley LC & Yaffe MB (2000) TAZ: a novel transcriptional co-activator regulated by interactions with 14-3-3 and PDZ domain proteins. *The EMBO journal* **19**: 6778–91
- Kanda T, Furuno A & Yoshiike K (1988) Human papillomavirus type 16 open reading frame E7 encodes a transforming gene for rat 3Y1 cells. *Journal of virology* **62**: 610–3
- Kang T, Wei Y, Honaker Y, Yamaguchi H, Appella E, Hung M-C & Piwnicka-Worms H (2008) GSK-3 beta targets Cdc25A for ubiquitin-mediated proteolysis, and GSK-3 beta inactivation correlates with Cdc25A overproduction in human cancers. *Cancer cell* **13**: 36–47
- Kasahara K, Goto H, Enomoto M, Tomono Y, Kiyono T & Inagaki M (2010) 14-3-3gamma mediates Cdc25A proteolysis to block premature mitotic entry after DNA damage. *Embo J* **29**: 2802–2812
- Kaur P & McDougall JK (1988) Characterization of primary human keratinocytes transformed by human papillomavirus type 18. *Journal of virology* **62**: 1917–24
- Keshamouni VG, Michailidis G, Grasso CS, Anthwal S, Strahler JR, Walker A, Arenberg DA, Reddy RC, Akulapalli S, Thannickal VJ, Standiford TJ, Andrews PC & Omenn GS (2006) Differential protein expression profiling



- by iTRAQ-2DLC-MS/MS of lung cancer cells undergoing epithelial-mesenchymal transition reveals a migratory/invasive phenotype. *J Proteome Res* **5**: 1143–1154
- Kessis TD, Connolly DC, Hedrick L & Cho KR (1996) Expression of HPV16 E6 or E7 increases integration of foreign DNA. *Oncogene* **13**: 427–31
- Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL & Shay JW (1994) Specific association of human telomerase activity with immortal cells and cancer. *Science (New York, N.Y.)* **266**: 2011–5
- Kim Y-N, Koo KH, Sung JY, Yun U-J & Kim H (2012) Anoikis resistance: an essential prerequisite for tumor metastasis. *International journal of cell biology* **2012**: 306879
- Kimple RJ, Smith MA, Blitzer GC, Torres AD, Martin JA, Yang RZ, Peet CR, Lorenz LD, Nickel KP, Klingelutz AJ, Lambert PF & Harari PM (2013) Enhanced radiation sensitivity in HPV-positive head and neck cancer. *Cancer research* **73**: 4791–800
- Kirnbauer R, Booy F, Cheng N, Lowy DR & Schiller JT (1992) Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. *Proceedings of the National Academy of Sciences of the United States of America* **89**: 12180–4
- Kirnbauer R, Taub J, Greenstone H, Roden R, Dürst M, Gissmann L, Lowy DR & Schiller JT (1993) Efficient self-assembly of human papillomavirus type 16 L1 and L1-L2 into virus-like particles. *Journal of virology* **67**: 6929–36
- Kiyono T, Foster SA, Koop JJ, McDougall JK, Galloway DA & Klingelutz AJ (1998) Both Rb/p16INK4a inactivation and telomerase activity are required to immortalize human epithelial cells. *Nature* **396**: 84–8
- Kiyono T, Hiraiwa A, Fujita M, Hayashi Y, Akiyama T & Ishibashi M (1997) Binding of high-risk human papillomavirus E6 oncoproteins to the human homologue of the Drosophila discs large tumor suppressor protein. *Proceedings of the National Academy of Sciences of the United States of America* **94**: 11612–6
- Kiyono T, Hiraiwa A, Ishii S, Takahashi T & Ishibashi M (1994) Inhibition of p53-mediated transactivation by E6 of type 1, but not type 5, 8, or 47, human papillomavirus of cutaneous origin. *Journal of virology* **68**: 4656–61

- Klingelhutz AJ, Barber SA, Smith PP, Dyer K & McDougall JK (1994) Restoration of telomeres in human papillomavirus-immortalized human anogenital epithelial cells. *Molecular and cellular biology* **14**: 961–9
- Klingelhutz AJ, Foster SA & McDougall JK (1996) Telomerase activation by the E6 gene product of human papillomavirus type 16. *Nature* **380**: 79–82
- Von Knebel Doeberitz M (2002) New markers for cervical dysplasia to visualise the genomic chaos created by aberrant oncogenic papillomavirus infections. *European journal of cancer (Oxford, England: 1990)* **38**: 2229–42
- Knight GL, Pugh AG, Yates E, Bell I, Wilson R, Moody CA, Laimins LA & Roberts S (2011) A cyclin-binding motif in human papillomavirus type 18 (HPV18) E1<sup>E4</sup> is necessary for association with CDK-cyclin complexes and G2/M cell cycle arrest of keratinocytes, but is not required for differentiation-dependent viral genome amplification or L1 cap. *Virology* **412**: 196–210
- Koff A, Giordano A, Desai D, Yamashita K, Harper J, Elledge S, Nishimoto T, Morgan D, Franza B & Roberts J (1992) Formation and activation of a cyclin E-cdk2 complex during the G1 phase of the human cell cycle. *Science* **257**: 1689–1694
- Kolch W, Heidecker G, Kochs G, Hummel R, Vahidi H, Mischak H, Finkenzeller G, Marmé D & Rapp UR (1993) Protein kinase C alpha activates RAF-1 by direct phosphorylation. *Nature* **364**: 249–52
- Konishi-Imamura L, Noda M & Nomura Y (1987) Alteration by v-Ki-ras in NaF, cholera toxin and forskolin-induced adenylate cyclase activation in NIH/3T3 fibroblast cells. *Biochemical and biophysical research communications* **146**: 47–52
- Konishi-Imamura L, Tohda M, Noda M, Nakano H & Nomura Y (1988) GTP-binding proteins and adenylate cyclase activity in v-Ki-ras transformed NIH/3T3 fibroblast cells. *Biochemical and biophysical research communications* **153**: 1214–22
- Kossatz U, Vervoorts J, Nicleleit I, Sundberg HA, Arthur JSC, Manns MP & Malek NP (2006) C-terminal phosphorylation controls the stability and function of p27kip1. *The EMBO journal* **25**: 5159–70
- Kotelevets L, van Hengel J, Bruyneel E, Mareel M, van Roy F & Chastre E (2005) Implication of the MAGI-1b/PTEN signalosome in stabilization of adherens junctions and suppression of invasiveness. *FASEB journal* :

*official publication of the Federation of American Societies for Experimental Biology* **19**: 115–7

- Kraemer L & Clement S (2014) Phosphorylation-independent recruitment of 14-3-3 alters tristetraprolin function (748.3). *FASEB J* **28**: 748.3–
- Kranjec C & Banks L (2011) A systematic analysis of human papillomavirus (HPV) E6 PDZ substrates identifies MAGI-1 as a major target of HPV type 16 (HPV-16) and HPV-18 whose loss accompanies disruption of tight junctions. *Journal of virology* **85**: 1757–64
- Kranjec C, Massimi P & Banks L (2014) Restoration of MAGI-1 Expression in HPV Positive Tumour Cells Induces Cell Growth Arrest and Apoptosis. *Journal of virology*
- Kühne C, Gardiol D, Guarnaccia C, Amenitsch H & Banks L (2000) Differential regulation of human papillomavirus E6 by protein kinase A: conditional degradation of human discs large protein by oncogenic E6. *Oncogene* **19**: 5884–5891
- Kumagai A & Dunphy WG (1999) Binding of 14-3-3 proteins and nuclear export control the intracellular localization of the mitotic inducer Cdc25. *Genes & development* **13**: 1067–72
- Kumar A, Zhao Y, Meng G, Zeng M, Srinivasan S, Delmolino LM, Gao Q, Dimri G, Weber GF, Wazer DE, Band H & Band V (2002) Human papillomavirus oncoprotein E6 inactivates the transcriptional coactivator human ADA3. *Molecular and cellular biology* **22**: 5801–12
- Kupryjańczyk J (1990) Epidermal growth factor receptor expression in the normal and inflamed cervix uteri: a comparison with estrogen receptor expression. *International journal of gynecological pathology: official journal of the International Society of Gynecological Pathologists* **9**: 263–71
- Kyo S, Klumpp DJ, Inoue M, Kanaya T & Laimins LA (1997) Expression of AP1 during cellular differentiation determines human papillomavirus E6/E7 expression in stratified epithelial cells. *J Gen Virol* **78** ( Pt 2): 401–411
- Lammer C, Wagerer S, Saffrich R, Mertens D, Ansorge W & Hoffmann I (1998) The cdc25B phosphatase is essential for the G2/M phase transition in human cells. *Journal of cell science* **111** ( Pt 1): 2445–53

- Laronga C, Yang HY, Neal C & Lee MH (2000) Association of the cyclin-dependent kinases and 14-3-3 sigma negatively regulates cell cycle progression. *J Biol Chem* **275**: 23106–23112
- Lechner MS, Mack DH, Finicle AB, Crook T, Vousden KH & Laimins LA (1992) Human papillomavirus E6 proteins bind p53 in vivo and abrogate p53-mediated repression of transcription. *The EMBO journal* **11**: 3045–52
- Lee C & Laimins LA (2004) Role of the PDZ domain-binding motif of the oncoprotein E6 in the pathogenesis of human papillomavirus type 31. *J Virol* **78**: 12366–12377
- Lee JO, Russo AA & Pavletich NP (1998) Structure of the retinoblastoma tumour-suppressor pocket domain bound to a peptide from HPV E7. *Nature* **391**: 859–65
- Lee SS, Weiss RS & Javier RT (1997) Binding of human virus oncoproteins to hDlg/SAP97, a mammalian homolog of the Drosophila discs large tumor suppressor protein. *Proceedings of the National Academy of Sciences of the United States of America* **94**: 6670–5
- Leffers H, Madsen P, Rasmussen HH, Honoré B, Andersen AH, Walbum E, Vandekerckhove J & Celis JE (1993) Molecular cloning and expression of the transformation sensitive epithelial marker stratifin. A member of a protein family that has been involved in the protein kinase C signalling pathway. *Journal of molecular biology* **231**: 982–98
- Legouis R, Jaulin-Bastard F, Schott S, Navarro C, Borg J-P & Labouesse M (2003) Basolateral targeting by leucine-rich repeat domains in epithelial cells. *EMBO reports* **4**: 1096–102
- Lengauer C, Kinzler KW & Vogelstein B (1997) Genetic instability in colorectal cancers. *Nature* **386**: 623–7
- Li N, Franceschi S, Howell-Jones R, Snijders PJF & Clifford GM (2011a) Human papillomavirus type distribution in 30,848 invasive cervical cancers worldwide: Variation by geographical region, histological type and year of publication. *International journal of cancer. Journal international du cancer* **128**: 927–35
- Li S, Janosch P, Tanji M, Rosenfeld GC, Waymire JC, Mischak H, Kolch W & Sedivy JM (1995) Regulation of Raf-1 kinase activity by the 14-3-3 family of proteins. *The EMBO journal* **14**: 685–96
- Li S, Labrecque S, Gauzzi MC, Cuddihy AR, Wong AH, Pellegrini S, Matlashewski GJ & Koromilas AE (1999) The human papilloma virus

- (HPV)-18 E6 oncoprotein physically associates with Tyk2 and impairs Jak-STAT activation by interferon-alpha. *Oncogene* **18**: 5727–37
- Li X & Coffino P (1996) High-risk human papillomavirus E6 protein has two distinct binding sites within p53, of which only one determines degradation. *Journal of virology* **70**: 4509–16
- Li X, Yang H, Liu J, Schmidt MD & Gao T (2011b) Scribble-mediated membrane targeting of PHLPP1 is required for its negative regulation of Akt. *EMBO reports* **12**: 818–24
- Light Y, Paterson H & Marais R (2002) 14-3-3 antagonizes Ras-mediated Raf-1 recruitment to the plasma membrane to maintain signaling fidelity. *Molecular and cellular biology* **22**: 4984–96
- Liu D, Bienkowska J, Petosa C, Collier RJ, Fu H & Liddington R (1995) Crystal structure of the zeta isoform of the 14-3-3 protein. *Nature* **376**: 191–194
- Liu P, Begley M, Michowski W, Inuzuka H, Ginzberg M, Gao D, Tsou P, Gan W, Papa A, Kim BM, Wan L, Singh A, Zhai B, Yuan M, Wang Z, Gygi SP, Lee TH, Lu K-P, Toker A, Pandolfi PP, et al (2014) Cell-cycle-regulated activation of Akt kinase by phosphorylation at its carboxyl terminus. *Nature* **508**: 541–5
- Liu Q, Guntuku S, Cui XS, Matsuoka S, Cortez D, Tamai K, Luo G, Carattini-Rivera S, DeMayo F, Bradley A, Donehower LA & Elledge SJ (2000) Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. *Genes & development* **14**: 1448–59
- Liu T-A, Jan Y-J, Ko B-S, Liang S-M, Chen S-C, Wang J, Hsu C, Wu Y-M & Liou J-Y (2013) 14-3-3ε overexpression contributes to epithelial-mesenchymal transition of hepatocellular carcinoma. *PloS one* **8**: e57968
- Liu X, Clements A, Zhao K & Marmorstein R (2006) Structure of the human Papillomavirus E7 oncoprotein and its mechanism for inactivation of the retinoblastoma tumor suppressor. *The Journal of biological chemistry* **281**: 578–86
- Liu X, Dakic A, Zhang Y, Dai Y, Chen R & Schlegel R (2009) HPV E6 protein interacts physically and functionally with the cellular telomerase complex. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 18780–5

- Liu Y, Li JZ, Yuan XH, Adler-Storthz K & Che Z (2002) An AP-1 binding site mutation in HPV-16 LCR enhances E6/E7 promoter activity in human oral epithelial cells. *Virus Genes* **24**: 29–37
- Lodygin D & Hermeking H (2005) The role of epigenetic inactivation of 14-3-3sigma in human cancer. *Cell Res* **15**: 237–246
- Logsdon CD, Simeone DM, Binkley C, Arumugam T, Greenson JK, Giordano TJ, Misek DE, Kuick R & Hanash S (2003) Molecular profiling of pancreatic adenocarcinoma and chronic pancreatitis identifies multiple genes differentially regulated in pancreatic cancer. *Cancer research* **63**: 2649–57
- Lopez-Girona A, Furnari B, Mondesert O & Russell P (1999) Nuclear localization of Cdc25 is regulated by DNA damage and a 14-3-3 protein. *Nature* **397**: 172–5
- Lorenz LD, Rivera Cardona J & Lambert PF (2013) Inactivation of p53 rescues the maintenance of high risk HPV DNA genomes deficient in expression of E6. *PLoS pathogens* **9**: e1003717
- Lu P., Zhou Z., Shen M & Lu P. (1999) Function of WW Domains as Phosphoserine- or Phosphothreonine-Binding Modules. *Science* **283**: 1325–1328
- Lu Z, Hu X, Li Y, Zheng L, Zhou Y, Jiang H, Ning T, Basang Z, Zhang C & Ke Y (2004a) Human papillomavirus 16 E6 oncoprotein interferences with insulin signaling pathway by binding to tuberlin. *J Biol Chem* **279**: 35664–35670
- Lu Z, Hu X, Li Y, Zheng L, Zhou Y, Jiang H, Ning T, Basang Z, Zhang C & Ke Y (2004b) Human papillomavirus 16 E6 oncoprotein interferences with insulin signaling pathway by binding to tuberlin. *The Journal of biological chemistry* **279**: 35664–70
- Lüscher-Firzlaff JM, Westendorf JM, Zwicker J, Burkhardt H, Henriksson M, Müller R, Pirollet F & Lüscher B (1999) Interaction of the fork head domain transcription factor MPP2 with the human papilloma virus 16 E7 protein: enhancement of transformation and transactivation. *Oncogene* **18**: 5620–30
- Lutzner MA, Blanchet-Bardon C & Orth G (1984) Clinical observations, virologic studies, and treatment trials in patients with epidermodysplasia verruciformis, a disease induced by specific human papillomaviruses. *The Journal of investigative dermatology* **83**: 18s–25s

- Macara IG (2004) Par Proteins: Partners in Polarization. *Current Biology* **14**: R160–R162
- Mackintosh C (2004) Dynamic interactions between 14-3-3 proteins and phosphoproteins regulate diverse cellular processes. *The Biochemical journal* **381**: 329–42
- Maiti GP, Mondal P, Mukherjee N, Ghosh A, Ghosh S, Dey S, Chakrabarty J, Roy A, Biswas J, Roychoudhury S & Panda CK (2013) Overexpression of EGFR in head and neck squamous cell carcinoma is associated with inactivation of SH3GL2 and CDC25A genes. *PloS one* **8**: e63440
- Mann DJ & Jones NC (1996) E2F-1 but not E2F-4 can overcome p16-induced G1 cell-cycle arrest. *Current Biology* **6**: 474–483
- Mannhardt B, Weinzimmer SA, Wagner M, Fiedler M, Cohen P, Jansen-Dürr P & Zwerschke W (2000) Human papillomavirus type 16 E7 oncoprotein binds and inactivates growth-inhibitory insulin-like growth factor binding protein 3. *Molecular and cellular biology* **20**: 6483–95
- Mantovani F & Banks L (2001) The human papillomavirus E6 protein and its contribution to malignant progression. *Oncogene* **20**: 7874–87
- Marais R, Light Y, Paterson HF & Marshall CJ (1995) Ras recruits Raf-1 to the plasma membrane for activation by tyrosine phosphorylation. *The EMBO journal* **14**: 3136–45
- Margolis SS, Perry JA, Forester CM, Nutt LK, Guo Y, Jardim MJ, Thomenius MJ, Freel CD, Darbandi R, Ahn J-H, Arroyo JD, Wang X-F, Shenolikar S, Nairn AC, Dunphy WG, Hahn WC, Virshup DM & Kornbluth S (2006) Role for the PP2A/B56delta phosphatase in regulating 14-3-3 release from Cdc25 to control mitosis. *Cell* **127**: 759–73
- Massimi P & Banks L (2000) Differential phosphorylation of the HPV-16 E7 oncoprotein during the cell cycle. *Virology* **276**: 388–94
- Massimi P, Narayan N, Cuenda A & Banks L (2006) Phosphorylation of the discs large tumour suppressor protein controls its membrane localisation and enhances its susceptibility to HPV E6-induced degradation. *Oncogene* **25**: 4276–4285
- Massimi P, Narayan N, Thomas M, Gammoh N, Strand S, Strand D & Banks L (2008) Regulation of the hDlg/hScrib/Hugl-1 tumour suppressor complex. *Experimental Cell Research* **314**: 3306–3317

- Massimi P, Pim D, Storey A & Banks L (1996) HPV-16 E7 and adenovirus E1a complex formation with TATA box binding protein is enhanced by casein kinase II phosphorylation. *Oncogene* **12**: 2325–2330
- Masters SC, Pederson KJ, Zhang L, Barbieri JT & Fu H (1999) Interaction of 14-3-3 with a nonphosphorylated protein ligand, exoenzyme S of *Pseudomonas aeruginosa*. *Biochemistry* **38**: 5216–21
- Matlashewski G, Schneider J, Banks L, Jones N, Murray A & Crawford L (1987) Human papillomavirus type 16 DNA cooperates with activated ras in transforming primary cells. *The EMBO journal* **6**: 1741–6
- Matsubara A, Sekine S, Ogawa R, Yoshida M, Kasamatsu T, Tsuda H & Kanai Y (2014) Lobular endocervical glandular hyperplasia is a neoplastic entity with frequent activating GNAS mutations. *The American journal of surgical pathology* **38**: 370–6
- Matsukura T, Koi S & Sugase M (1989) Both episomal and integrated forms of human papillomavirus type 16 are involved in invasive cervical cancers. *Virology* **172**: 63–72
- Matsumine A, Ogai A, Senda T, Okumura N, Satoh K, Baeg G-H, Kawahara T, Kobayashi S, Okada M, Toyoshima K & Akiyama T (1996) Binding of APC to the Human Homolog of the Drosophila Discs Large Tumor Suppressor Protein. *Science* **272**: 1020–1023
- Matta A, Bahadur S, Duggal R, Gupta SD & Ralhan R (2007) Over-expression of 14-3-3zeta is an early event in oral cancer. *BMC Cancer* **7**: 169
- Mazzarelli JM, Atkins GB, Geisberg J V & Ricciardi RP (1995) The viral oncoproteins Ad5 E1A, HPV16 E7 and SV40 TAg bind a common region of the TBP-associated factor-110. *Oncogene* **11**: 1859–64
- McBride AA, Oliveira JG & McPhillips MG (2006) Partitioning viral genomes in mitosis: same idea, different targets. *Cell cycle (Georgetown, Tex.)* **5**: 1499–502
- McIntosh PB, Laskey P, Sullivan K, Davy C, Wang Q, Jackson DJ, Griffin HM & Doorbar J (2010) E1–E4-mediated keratin phosphorylation and ubiquitylation: a mechanism for keratin depletion in HPV16-infected epithelium. *Journal of cell science* **123**: 2810–22
- McIntosh PB, Martin SR, Jackson DJ, Khan J, Isaacson ER, Calder L, Raj K, Griffin HM, Wang Q, Laskey P, Eccleston JF & Doorbar J (2008) Structural analysis reveals an amyloid form of the human papillomavirus



- type 16 E1--E4 protein and provides a molecular basis for its accumulation. *Journal of virology* **82**: 8196–203
- McIntyre MC, Frattini MG, Grossman SR & Laimins LA (1993) Human papillomavirus type 18 E7 protein requires intact Cys-X-X-Cys motifs for zinc binding, dimerization, and transformation but not for Rb binding. *Journal of virology* **67**: 3142–50
- McMillan NA, Payne E, Frazer IH & Evander M (1999) Expression of the alpha6 integrin confers papillomavirus binding upon receptor-negative B-cells. *Virology* **261**: 271–9
- McMurray HR, Nguyen D, Westbrook TF & McAnce DJ (2001) Biology of human papillomaviruses. *Int J Exp Pathol* **82**: 15–33
- McPherson RA, Harding A, Roy S, Lane A & Hancock JF (1999) Interactions of c-Raf-1 with phosphatidylserine and 14-3-3. *Oncogene* **18**: 3862–3869
- Mechler BM, McGinnis W & Gehring WJ (1985) Molecular cloning of lethal(2)giant larvae, a recessive oncogene of *Drosophila melanogaster*. *The EMBO journal* **4**: 1551–7
- Meek DW (1994) Post-translational modification of p53. *Seminars in cancer biology* **5**: 203–10
- Megidish T, Cooper J, Zhang L, Fu H & Hakomori S (1998) A novel sphingosine-dependent protein kinase (SDK1) specifically phosphorylates certain isoforms of 14-3-3 protein. *The Journal of biological chemistry* **273**: 21834–45
- Menges CW, Baglia LA, Lapoint R & McCance DJ (2006) Human papillomavirus type 16 E7 up-regulates AKT activity through the retinoblastoma protein. *Cancer research* **66**: 5555–9
- Meyers C & Laimins LA (1994) In vitro systems for the study and propagation of human papillomaviruses. *Current topics in microbiology and immunology* **186**: 199–215
- Meyers JM, Spangle JM & Munger K (2013) The human papillomavirus type 8 E6 protein interferes with NOTCH activation during keratinocyte differentiation. *Journal of virology* **87**: 4762–7
- Mhawech P (2005) 14-3-3 proteins-an update. *Cell Research* **15**: 228–236
- Michaud NR, Fabian JR, Mathes KD & Morrison DK (1995) 14-3-3 is not essential for Raf-1 function: identification of Raf-1 proteins that are

- biologically activated in a 14-3-3- and Ras-independent manner. *Mol Cell Biol* **15**: 3390–3397
- Middleton K, Peh W, Southern S, Griffin H, Sotlar K, Nakahara T, El-Sherif A, Morris L, Seth R, Hibma M, Jenkins D, Lambert P, Coleman N & Doorbar J (2003) Organization of Human Papillomavirus Productive Cycle during Neoplastic Progression Provides a Basis for Selection of Diagnostic Markers. *Journal of Virology* **77**: 10186–10201
- Modis Y, Trus BL & Harrison SC (2002) Atomic model of the papillomavirus capsid. *The EMBO journal* **21**: 4754–62
- Mohammad DK, Nore BF, Hussain A, Gustafsson MO, Mohamed AJ & Smith CIE (2013) Dual phosphorylation of Btk by Akt/protein kinase b provides docking for 14-3-3 $\zeta$ , regulates shuttling, and attenuates both tonic and induced signaling in B cells. *Molecular and cellular biology* **33**: 3214–26
- Molzan M & Ottmann C (2012) Synergistic binding of the phosphorylated S233- and S259-binding sites of C-RAF to one 14-3-3 $\zeta$  dimer. *Journal of molecular biology* **423**: 486–95
- Molzan M, Schumacher B, Ottmann C, Baljuls A, Polzien L, Weyand M, Thiel P, Rose R, Rose M, Kuhenne P, Kaiser M, Rapp UR, Kuhlmann J & Ottmann C (2010) Impaired binding of 14-3-3 to C-RAF in Noonan syndrome suggests new approaches in diseases with increased Ras signaling. *Molecular and cellular biology* **30**: 4698–711
- Moody CA & Laimins LA (2009) Human papillomaviruses activate the ATM DNA damage pathway for viral genome amplification upon differentiation. *PLoS pathogens* **5**: e1000605
- Moore BW & McGregor D (1965) Chromatographic and electrophoretic fractionation of soluble proteins of brain and liver. *The Journal of biological chemistry* **240**: 1647–53
- Moore BW. & Perez VJ (1967) Specific acidic proteins of the nervous system. In *Physiological and Biochemical Aspects of Nervous Integration*, Carlson FD (ed) pp 343 – 59. Englewood Cliffs, NJ: Prentice- Hall
- Moreira JMA, Gromov P & Celis JE (2004) Expression of the tumor suppressor protein 14-3-3 sigma is down-regulated in invasive transitional cell carcinomas of the urinary bladder undergoing epithelial-to-mesenchymal transition. *Molecular & cellular proteomics : MCP* **3**: 410–9

- Morgan D, Pecoraro G, Rosenberg I & Defendi V (1988) Transformation of C127 mouse fibroblasts by human papillomavirus 16. *Cancer research* **48**: 2505–11
- Morrison DK, Heidecker G, Rapp UR & Copeland TD (1993) Identification of the major phosphorylation sites of the Raf-1 kinase. *The Journal of biological chemistry* **268**: 17309–16
- Münger K, Baldwin A, Edwards KM, Hayakawa H, Nguyen CL, Owens M, Grace M & Huh K (2004) Mechanisms of human papillomavirus-induced oncogenesis. *Journal of virology* **78**: 11451–60
- Münger K, Basile JR, Duensing S, Eichten A, Gonzalez SL, Grace M & Zacny VL (2001) Biological activities and molecular targets of the human papillomavirus E7 oncoprotein. *Oncogene* **20**: 7888–98
- Munger K & Howley PM (2002) Human papillomavirus immortalization and transformation functions. *Virus Res* **89**: 213–228
- Münger K, Werness BA, Dyson N, Phelps WC, Harlow E & Howley PM (1989) Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. *The EMBO journal* **8**: 4099–105
- Muslin AJ, Tanner JW, Allen PM & Shaw AS (1996) Interaction of 14-3-3 with Signaling Proteins Is Mediated by the Recognition of Phosphoserine. *Cell* **84**: 889–897
- Muslin AJ & Xing H (2000) 14-3-3 proteins: regulation of subcellular localization by molecular interference. *Cellular Signalling* **12**: 703–709
- Nagasaka K, Pim D, Massimi P, Thomas M, Tomaić V, Subbaiah VK, Kranjec C, Nakagawa S, Yano T, Taketani Y, Myers M & Banks L (2010) The cell polarity regulator hScrib controls ERK activation through a KIM site-dependent interaction. *Oncogene* **29**: 5311–21
- Nakagawa S & Huibregtse JM (2000) Human scribble (Vartul) is targeted for ubiquitin-mediated degradation by the high-risk papillomavirus E6 proteins and the E6AP ubiquitin-protein ligase. *Mol Cell Biol* **20**: 8244–8253
- Nakagawa S, Watanabe S, Yoshikawa H, Taketani Y, Yoshiike K & Kanda T (1995) Mutational analysis of human papillomavirus type 16 E6 protein: transforming function for human cells and degradation of p53 in vitro. *Virology* **212**: 535–42

- Nakahara T, Nishimura A, Tanaka M, Ueno T, Ishimoto A & Sakai H (2002) Modulation of the cell division cycle by human papillomavirus type 18 E4. *Journal of virology* **76**: 10914–20
- Narayan N, Massimi P & Banks L (2009a) CDK phosphorylation of the discs large tumour suppressor controls its localisation and stability. *Journal of cell science* **122**: 65–74
- Narayan N, Subbaiah VK & Banks L (2009b) The high-risk HPV E6 oncoprotein preferentially targets phosphorylated nuclear forms of hDlg. *Virology* **387**: 1–4
- Nasseri M, Hirochika R, Broker TR & Chow LT (1987) A human papilloma virus type 11 transcript encoding an E1<sup>E4</sup> protein. *Virology* **159**: 433–9
- Neal CL, Yao J, Yang W, Zhou X, Nguyen NT, Lu J, Danes CG, Guo H, Lan K-H, Ensor J, Hittelman W, Hung M-C & Yu D (2009) 14-3-3 $\zeta$  Overexpression Defines High Risk for Breast Cancer Recurrence and Promotes Cancer Cell Survival. *Cancer Research* **69**: 3425–32
- Neal CL & Yu D (2010) 14-3-3 $\zeta$  as a prognostic marker and therapeutic target for cancer. *Expert Opin Ther Targets* **12**:1343 - 53
- Nees M, Geoghegan JM, Munson P, Prabhu V, Liu Y, Androphy E & Woodworth CD (2000) Human Papillomavirus Type 16 E6 and E7 Proteins Inhibit Differentiation-dependent Expression of Transforming Growth Factor- $\beta$ 2 in Cervical Keratinocytes. *Cancer Res.* **60**: 4289–4298
- Nguyen CL, Eichwald C, Nibert ML & Münger K (2007) Human papillomavirus type 16 E7 oncoprotein associates with the centrosomal component gamma-tubulin. *Journal of virology* **81**: 13533–43
- Nguyen CL, McLaughlin-Drubin ME & Münger K (2008) Delocalization of the microtubule motor Dynein from mitotic spindles by the human papillomavirus E7 oncoprotein is not sufficient for induction of multipolar mitoses. *Cancer research* **68**: 8715–22
- Nguyen CL & Münger K (2009) Human papillomavirus E7 protein deregulates mitosis via an association with nuclear mitotic apparatus protein 1. *Journal of virology* **83**: 1700–7
- Nguyen DX, Westbrook TF & McCance DJ (2002a) Human Papillomavirus Type 16 E7 Maintains Elevated Levels of the cdc25A Tyrosine Phosphatase during Deregulation of Cell Cycle Arrest. *Journal of Virology* **76**: 619–632

- Nguyen M, Song S, Liem A, Androphy E, Liu Y & Lambert PF (2002b) A mutant of human papillomavirus type 16 E6 deficient in binding alpha-helix partners displays reduced oncogenic potential in vivo. *J Virol* **76**: 13039–13048
- Nguyen ML, Nguyen MM, Lee D, Griep AE & Lambert PF (2003) The PDZ ligand domain of the human papillomavirus type 16 E6 protein is required for E6's induction of epithelial hyperplasia in vivo. *J Virol* **77**: 6957–6964
- Nicolaides L, Davy C, Raj K, Kranjec C, Banks L & Doorbar J (2011) Stabilization of HPV16 E6 protein by PDZ proteins, and potential implications for genome maintenance. *Virology* **414**: 137–145
- Nominé Y, Masson M, Charbonnier S, Zanier K, Ristriani T, Deryckère F, Sibling A-P, Desplancq D, Atkinson RA, Weiss E, Orfanoudakis G, Kieffer B & Travé G (2006) Structural and functional analysis of E6 oncoprotein: insights in the molecular pathways of human papillomavirus-mediated pathogenesis. *Molecular cell* **21**: 665–78
- Nomura M, Shimizu S, Ito T, Narita M, Matsuda H & Tsujimoto Y (1999) Apoptotic Cytosol Facilitates Bax Translocation to Mitochondria That Involves Cytosolic Factor Regulated by Bcl-2. *Cancer Res.* **59**: 5542–5548
- Nyberg KA, Michelson RJ, Putnam CW & Weinert TA (2002) Toward maintaining the genome: DNA damage and replication checkpoints. *Annual review of genetics* **36**: 617–56
- O'Toole TE, Bialkowska K, Li X & Fox JEB (2011) Tiam1 is recruited to  $\beta$ 1-integrin complexes by 14-3-3 $\zeta$  where it mediates integrin-induced Rac1 activation and motility. *Journal of cellular physiology* **226**: 2965–78
- Obata T, Yaffe MB, Leparo GG, Piro ET, Maegawa H, Kashiwagi A, Kikkawa R & Cantley LC (2000) Peptide and protein library screening defines optimal substrate motifs for AKT/PKB. *The Journal of Biological Chemistry* **275**: 36108–36115
- Obenauer JC, Cantley LC & Yaffe MB (2003) Scansite 2.0: Proteome-wide prediction of cell signaling interactions using short sequence motifs. *Nucleic acids research* **31**: 3635–41
- Obsil T, Ghirlando R, Klein DC, Ganguly S & Dyda F (2001) Crystal structure of the 14-3-3 $\zeta$ :serotonin N-acetyltransferase complex. a role for scaffolding in enzyme regulation. *Cell* **105**: 257–67

- Obsilova V, Vecer J, Herman P, Pabianova A, Sulc M, Teisinger J, Boura E & Obsil T (2005) 14-3-3 Protein interacts with nuclear localization sequence of forkhead transcription factor FoxO4. *Biochemistry* **44**: 11608–17
- Oh K-J, Kalinina A, Wang J, Nakayama K, Nakayama KI & Bagchi S (2004a) The papillomavirus E7 oncoprotein is ubiquitinated by UbcH7 and Cullin 1- and Skp2-containing E3 ligase. *Journal of virology* **78**: 5338–46
- Oh S, Shin S, Lightfoot SA & Janknecht R (2013) 14-3-3 proteins modulate the ETS transcription factor ETV1 in prostate cancer. *Cancer research* **73**: 5110–9
- Oh ST, Kyo S & Laimins LA (2001) Telomerase activation by human papillomavirus type 16 E6 protein: induction of human telomerase reverse transcriptase expression through Myc and GC-rich Sp1 binding sites. *Journal of virology* **75**: 5559–66
- Oh ST, Longworth MS & Laimins LA (2004b) Roles of the E6 and E7 proteins in the life cycle of low-risk human papillomavirus type 11. *J Virol* **78**: 2620–2626
- Ohlenschläger O, Seiboth T, Zengerling H, Briesse L, Marchanka A, Ramachandran R, Baum M, Korbass M, Meyer-Klaucke W, Dürst M & Görlach M (2006) Solution structure of the partially folded high-risk human papilloma virus 45 oncoprotein E7. *Oncogene* **25**: 5953–9
- Okayama A, Miyagi Y, Oshita F, Nishi M, Nakamura Y, Nagashima Y, Akimoto K, Ryo A & Hirano H (2014) Proteomic Analysis of Proteins Related to Prognosis of Lung Adenocarcinoma. *Journal of proteome research*
- Orlicky S, Tang X, Willems A, Tyers M & Sicheri F (2003) Structural basis for phosphodependent substrate selection and orientation by the SCFCdc4 ubiquitin ligase. *Cell* **112**: 243–56
- Ostrow RS, LaBresh K V & Faras AJ (1991) Characterization of the complete RhPV 1 genomic sequence and an integration locus from a metastatic tumor. *Virology* **181**: 424–9
- Ostrow RS, McGlennen RC, Shaver MK, Kloster BE, Houser D & Faras AJ (1990) A rhesus monkey model for sexual transmission of a papillomavirus isolated from a squamous cell carcinoma. *Proceedings of the National Academy of Sciences of the United States of America* **87**: 8170–4

- Ou Y-H, Chung P-H, Sun T-P & Shieh S-Y (2005) p53 C-terminal phosphorylation by CHK1 and CHK2 participates in the regulation of DNA-damage-induced C-terminal acetylation. *Molecular biology of the cell* **16**: 1684–95
- Ozbun MA & Meyers C (1998a) Temporal usage of multiple promoters during the life cycle of human papillomavirus type 31b. *Journal of virology* **72**: 2715–22
- Ozbun MA & Meyers C (1998b) Human papillomavirus type 31b E1 and E2 transcript expression correlates with vegetative viral genome amplification. *Virology* **248**: 218–30
- Padden J, Megger DA, Bracht T, Reis H, Ahrens M, Kohl M, Eisenacher M, Schlaak JF, Canbay AE, Weber F, Hoffmann A-C, Kuhlmann K, Meyer HE, Baba HA & Sitek B (2014) Identification of Novel Biomarker Candidates for the Immunohistochemical Diagnosis of Cholangiocellular Carcinoma. *Molecular & cellular proteomics : MCP*
- Parish JL, Bean AM, Park RB & Androphy EJ (2006) ChlR1 is required for loading papillomavirus E2 onto mitotic chromosomes and viral genome maintenance. *Molecular cell* **24**: 867–76
- Park JW, Shin M-K & Lambert PF (2014) High incidence of female reproductive tract cancers in FA-deficient HPV16-transgenic mice correlates with E7's induction of DNA damage response, an activity mediated by E7's inactivation of pocket proteins. *Oncogene* **33**: 3383–91
- Park RB & Androphy EJ (2002) Genetic analysis of high-risk e6 in episomal maintenance of human papillomavirus genomes in primary human keratinocytes. *J Virol* **76**: 11359–11364
- Patel D, Huang SM, Baglia LA & McCance DJ (1999) The E6 protein of human papillomavirus type 16 binds to and inhibits co-activation by CBP and p300. *The EMBO journal* **18**: 5061–72
- Pearson RB & Kemp BE (1991) Protein kinase phosphorylation site sequences and consensus specificity motifs: tabulations. *Methods in enzymology* **200**: 62–81
- Peng CY, Graves PR, Ogg S, Thoma RS, Byrnes MJ, Wu Z, Stephenson MT & Piwnica-Worms H (1998) C-TAK1 protein kinase phosphorylates human Cdc25C on serine 216 and promotes 14-3-3 protein binding. *Cell growth & differentiation: the molecular biology journal of the American Association for Cancer Research* **9**: 197–208

- Peng CY, Graves PR, Thoma RS, Wu Z, Shaw AS & Piwnica-Worms H (1997) Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. *Science (New York, N.Y.)* **277**: 1501–5
- Petosa C, Masters SC, Bankston LA, Pohl J, Wang B, Fu H & Liddington RC (1998) 14-3-3zeta binds a phosphorylated Raf peptide and an unphosphorylated peptide via its conserved amphipathic groove. *The Journal of biological chemistry* **273**: 16305–10
- Pett MR (2004) Acquisition of High-Level Chromosomal Instability Is Associated with Integration of Human Papillomavirus Type 16 in Cervical Keratinocytes. *Cancer Research* **64**: 1359–1368
- Pfister H (1992) Human papillomaviruses and skin cancer. *Seminars in cancer biology* **3**: 263–71
- Phelps WC, Münger K, Yee CL, Barnes JA & Howley PM (1992) Structure-function analysis of the human papillomavirus type 16 E7 oncoprotein. *Journal of virology* **66**: 2418–27
- Phelps WC, Yee CL, Münger K & Howley PM (1988) The human papillomavirus type 16 E7 gene encodes transactivation and transformation functions similar to those of adenovirus E1A. *Cell* **53**: 539–47
- Pietenpol JA, Stein RW, Moran E, Yaciuk P, Schlegel R, Lyons RM, Pittelkow MR, Münger K, Howley PM & Moses HL (1990) TGF- $\beta$ 1 inhibition of c-myc transcription and growth in keratinocytes is abrogated by viral transforming proteins with pRB binding domains. *Cell* **61**: 777–785
- Pim D & Banks L (2010) Interaction of viral oncoproteins with cellular target molecules: infection with high-risk vs low-risk human papillomaviruses. *APMIS: acta pathologica, microbiologica, et immunologica Scandinavica* **118**: 471–93
- Pim D, Bergant M, Boon SS, Ganti K, Kranjec C, Massimi P, Subbaiah VK, Thomas M, Tomaić V & Banks L (2012) Human papillomaviruses and the specificity of PDZ domain targeting. *The FEBS journal* **279**: 3530–7
- Pim D, Collins M & Banks L (1992) Human papillomavirus type 16 E5 gene stimulates the transforming activity of the epidermal growth factor receptor. *Oncogene* **7**: 27–32
- Pim D, Thomas M, Javier R, Gardiol D & Banks L (2000) HPV E6 targeted degradation of the discs large protein: evidence for the involvement of a novel ubiquitin ligase. *Oncogene* **19**: 719–725



- Pirami L, Giachè V & Becciolini A (1997) Analysis of HPV16, 18, 31, and 35 DNA in pre-invasive and invasive lesions of the uterine cervix. *Journal of clinical pathology* **50**: 600–4
- Pirisi L, Yasumoto S, Feller M, Doniger J & DiPaolo JA (1987) Transformation of human fibroblasts and keratinocytes with human papillomavirus type 16 DNA. *Journal of virology* **61**: 1061–6
- Pitot HC (1986) The molecular determinants of carcinogenesis. *Symposium on Fundamental Cancer Research* **39**: 187–96
- Plevin R, Malarkey K, Aidulis D, McLees A & Gould GW (1997) Cyclic AMP inhibitors inhibits PDGF-stimulated mitogen-activated protein kinase activity in rat aortic smooth muscle cells via inactivation of c-Raf-1 kinase and induction of MAP kinase phosphatase-1. *Cellular signalling* **9**: 323–8
- Vande Pol SB & Klingelhutz AJ (2013) Papillomavirus E6 oncoproteins. *Virology* **445**: 115–37
- Powell DW, Rane MJ, Chen Q, Singh S & McLeish KR (2002) Identification of 14-3-3zeta as a protein kinase B/Akt substrate. *The Journal of biological chemistry* **277**: 21639–42
- Powell DW, Rane MJ, Joughin BA, Kalmukova R, Hong J-H, Tidor B, Dean WL, Pierce WM, Klein JB, Yaffe MB & McLeish KR (2003) Proteomic Identification of 14-3-3 as a Mitogen-Activated Protein Kinase-Activated Protein Kinase 2 Substrate: Role in Dimer Formation and Ligand Binding. *Molecular and Cellular Biology* **23**: 5376–5387
- Prenzel N, Fischer OM, Streit S, Hart S & Ullrich A (2001) The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification. *Endocrine-related cancer* **8**: 11–31
- Prymakowska-Bosak M, Hock R, Catez F, Lim J-H, Birger Y, Shirakawa H, Lee K & Bustin M (2002) Mitotic phosphorylation of chromosomal protein HMGN1 inhibits nuclear import and promotes interaction with 14.3.3 proteins. *Molecular and cellular biology* **22**: 6809–19
- Pyeon D, Pearce SM, Lank SM, Ahlquist P & Lambert PF (2009) Establishment of human papillomavirus infection requires cell cycle progression. *PLoS pathogens* **5**: e1000318
- Quarmany VE & Korach KS (1984) The influence of 17 beta-estradiol on patterns of cell division in the uterus. *Endocrinology* **114**: 694–702

- Radhakrishnan VM & Martinez JD (2010) 14-3-3gamma induces oncogenic transformation by stimulating MAP kinase and PI3K signaling. *PloS one* **5**: e11433
- Rahman M, Lovat F, Romano G, Calore F, Acunzo M, Bell EH & Nana-Sinkam P (2014) miR-15b/16-2 regulates factors that promote p53 phosphorylation and augments the DNA damage response following radiation in the lung. *The Journal of biological chemistry*
- Rajagopalan S, Jaulent AM, Wells M, Veprintsev DB & Fersht AR (2008) 14-3-3 activation of DNA binding of p53 by enhancing its association into tetramers. *Nucleic acids research* **36**: 5983–91
- Rajagopalan S, Sade RS, Townsley FM & Fersht AR (2010) Mechanistic differences in the transcriptional activation of p53 by 14-3-3 isoforms. *Nucleic acids research* **38**: 893–906
- Rawls JA, Pusztai R & Green M (1990) Chemical synthesis of human papillomavirus type 16 E7 oncoprotein: autonomous protein domains for induction of cellular DNA synthesis and for trans activation. *J. Virol.* **64**: 6121–6129
- Ray D, Terao Y, Fuhrken PG, Ma Z-Q, DeMayo FJ, Christov K, Heerema NA, Franks R, Tsai SY, Papoutsakis ET & Kiyokawa H (2007) Deregulated CDC25A expression promotes mammary tumorigenesis with genomic instability. *Cancer research* **67**: 984–91
- Reich NC & Levine AJ (1984) Growth regulation of a cellular tumour antigen, p53, in nontransformed cells. *Nature* **308**: 199–201
- Reinstein E, Scheffner M, Oren M, Ciechanover A & Schwartz A (2000) Degradation of the E7 human papillomavirus oncoprotein by the ubiquitin-proteasome system: targeting via ubiquitination of the N-terminal residue. *Oncogene* **19**: 5944–50
- Ressler S, Scheiden R, Dreier K, Laich A, Müller-Holzner E, Pircher H, Morandell D, Stein I, Viertler H-P, Santer FR, Widschwendter A, Even J, Jansen-Dürr P, Capesius C & Zwerschke W (2007) High-risk human papillomavirus E7 oncoprotein detection in cervical squamous cell carcinoma. *Clinical cancer research: an official journal of the American Association for Cancer Research* **13**: 7067–72
- Riley RR, Duensing S, Brake T, Munger K, Lambert PF & Arbeit JM (2003) Dissection of Human Papillomavirus E6 and E7 Function in Transgenic Mouse Models of Cervical Carcinogenesis. *Cancer Res.* **63**: 4862–4871

- Rincon-Orozco B, Halec G, Rosenberger S, Muschik D, Nindl I, Bachmann A, Ritter TM, Dondog B, Ly R, Bosch FX, Zawatzky R & Rösl F (2009) Epigenetic silencing of interferon-kappa in human papillomavirus type 16-positive cells. *Cancer research* **69**: 8718–25
- Ristriani T, Nomine Y, Laurent C, Weiss E & Trave G (2002) Protein mutagenesis with monodispersity-based quality probing: selective inactivation of p53 degradation and DNA-binding properties of HPV E6 oncoprotein. *Protein Expr Purif* **26**: 357–367
- Ritt DA, Monson DM, Specht SI & Morrison DK (2010) Impact of feedback phosphorylation and Raf heterodimerization on normal and mutant B-Raf signaling. *Molecular and cellular biology* **30**: 806–19
- Rittinger K, Budman J, Xu J, Volinia S, Cantley LC, Smerdon SJ, Gamblin SJ & Yaffe MB (1999) Structural Analysis of 14-3-3 Phosphopeptide Complexes Identifies a Dual Role for the Nuclear Export Signal of 14-3-3 in Ligand Binding. *Molecular Cell* **4**: 153–166
- Roden RB, Day PM, Bronzo BK, Yutzy WH, Yang Y, Lowy DR & Schiller JT (2001) Positively charged termini of the L2 minor capsid protein are necessary for papillomavirus infection. *Journal of virology* **75**: 10493–7
- Roden RB, Lowy DR & Schiller JT (1997) Papillomavirus is resistant to desiccation. *The Journal of infectious diseases* **176**: 1076–9
- Roman A (2006) The Human Papillomavirus E7 Protein Shines a Spotlight on the pRB Family Member, p130. *Cell Cycle* **5**: 567–568
- Rommel C, Radziwill G, Lovrić J, Noeldeke J, Heinicke T, Jones D, Aitken A & Moelling K (1996) Activated Ras displaces 14-3-3 protein from the amino terminus of c-Raf-1. *Oncogene* **12**: 609–19
- Rommel C, Radziwill G, Moelling K & Hafen E (1997) Negative regulation of Raf activity by binding of 14-3-3 to the amino terminus of Raf in vivo. *Mechanisms of Development* **64**: 95–104
- Ronco L V, Karpova AY, Vidal M & Howley PM (1998) Human papillomavirus 16 E6 oncoprotein binds to interferon regulatory factor-3 and inhibits its transcriptional activity. *Genes & development* **12**: 2061–72
- Roy S, McPherson RA, Apolloni A, Yan J, Lane A, Clyde-Smith J & Hancock JF (1998) 14-3-3 Facilitates Ras-Dependent Raf-1 Activation In Vitro and In Vivo. *Mol. Cell. Biol.* **18**: 3947–3955

- Rudolph NS & Latt SA (1989) Flow cytometric analysis of X-ray sensitivity in ataxia telangiectasia. *Mutation research* **211**: 31–41
- Rushworth LK, Hindley AD, O'Neill E & Kolch W (2006) Regulation and role of Raf-1/B-Raf heterodimerization. *Molecular and cellular biology* **26**: 2262–72
- Russell P (1998) Checkpoints on the road to mitosis. *Trends in Biochemical Sciences* **23**: 399–402
- Sabatini DM (2006) mTOR and cancer: insights into a complex relationship. *Nature reviews. Cancer* **6**: 729–34
- Sagae S, Kuzumaki N, Hisada T, Mugikura Y, Kudo R & Hashimoto M (1989) ras oncogene expression and prognosis of invasive squamous cell carcinomas of the uterine cervix. *Cancer* **63**: 1577–82
- Saito S, Goodarzi AA, Higashimoto Y, Noda Y, Lees-Miller SP, Appella E & Anderson CW (2002) ATM mediates phosphorylation at multiple p53 sites, including Ser(46), in response to ionizing radiation. *The Journal of biological chemistry* **277**: 12491–4
- Sanchez Y, Wong C, Thoma RS, Richman R, Wu Z, Piwnica-Worms H & Elledge SJ (1997) Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25. *Science (New York, N.Y.)* **277**: 1497–501
- Sano T, Shimooka H, Weixa P, Segawa A, Jian Z, Motegi A, Nakayama H, Oyama T & Nakajima T (2004) Immunohistochemical expression of 14-3-3 sigma protein in various histological subtypes of uterine cervical cancers. *Pathology international* **54**: 743–50
- Santer FR, Moser B, Spoden GA, Jansen-Dürr P & Zwerschke W (2007) Human papillomavirus type 16 E7 oncoprotein inhibits apoptosis mediated by nuclear insulin-like growth factor-binding protein-3 by enhancing its ubiquitin/proteasome-dependent degradation. *Carcinogenesis* **28**: 2511–20
- Santoni M-J, Pontarotti P, Birnbaum D & Borg J-P (2002) The LAP family: a phylogenetic point of view. *Trends in genetics : TIG* **18**: 494–7
- Sapp M & Bienkowska-Haba M (2009) Viral entry mechanisms: human papillomavirus and a long journey from extracellular matrix to the nucleus. *The FEBS journal* **276**: 7206–16
- Sato H, Watanabe S, Furuno A & Yoshiike K (1989) Human papillomavirus type 16 E7 protein expressed in Escherichia coli and monkey COS-1 cells:

- immunofluorescence detection of the nuclear E7 protein. *Virology* **170**: 311–5
- Scheffner M, Huibregtse JM, Vierstra RD & Howley PM (1993) The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. *Cell* **75**: 495–505
- Scheffner M, Munger K, Byrne JC & Howley PM (1991) The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines. *Proc Natl Acad Sci U S A* **88**: 5523–5527
- Scheffner M, Werness BA, Huibregtse JM, Levine AJ & Howley PM (1990) The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. *Cell* **63**: 1129–1136
- Schlegel R, Phelps WC, Zhang YL & Barbosa M (1988) Quantitative keratinocyte assay detects two biological activities of human papillomavirus DNA and identifies viral types associated with cervical carcinoma. *The EMBO journal* **7**: 3181–7
- Schmitt A, Rochat A, Zeltner R, Borenstein L, Barrandon Y, Wettstein FO & Ifner T (1996) The primary target cells of the high-risk cottontail rabbit papillomavirus colocalize with hair follicle stem cells. *Journal of virology* **70**: 1912–22
- Schmitt JM & Stork PJ (2000) beta 2-adrenergic receptor activates extracellular signal-regulated kinases (ERKs) via the small G protein rap1 and the serine/threonine kinase B-Raf. *The Journal of biological chemistry* **275**: 25342–50
- Schneider MA, Spoden GA, Florin L & Lambert C (2011) Identification of the dynein light chains required for human papillomavirus infection. *Cellular microbiology* **13**: 32–46
- Schröder MS, Stellmacher A, Romorini S, Marini C, Montenegro-Venegas C, Altrock WD, Gundelfinger ED & Fejtova A (2013) Regulation of presynaptic anchoring of the scaffold protein Bassoon by phosphorylation-dependent interaction with 14-3-3 adaptor proteins. *PloS one* **8**: e58814
- Schwarz E, Freese UK, Gissmann L, Mayer W, Roggenbuck B, Stremlau A & Hausen H zur (1985) Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. *Nature* **314**: 111–114
- Seamon KB, Padgett W & Daly JW (1981) Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells.

- Proceedings of the National Academy of Sciences of the United States of America* **78**: 3363–7
- Sedman SA, Barbosa MS, Vass WC, Hubbert NL, Haas JA, Lowy DR & Schiller JT (1991) The full-length E6 protein of human papillomavirus type 16 has transforming and trans-activating activities and cooperates with E7 to immortalize keratinocytes in culture. *Journal of virology* **65**: 4860–6
- Seger R & Krebs EG (1995) The MAPK signaling cascade. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* **9**: 726–35
- Segrelles C, Lu J, Hammann B, Santos M, Moral M, Cascallana JL, Lara MF, Rho O, Carbajal S, Traag J, Beltrán L, Martínez-Cruz AB, García-Escudero R, Lorz C, Ruiz S, Bravo A, Paramio JM & DiGiovanni J (2007) Deregulated activity of Akt in epithelial basal cells induces spontaneous tumors and heightened sensitivity to skin carcinogenesis. *Cancer research* **67**: 10879–88
- Seimiya H, Sawada H, Muramatsu Y, Shimizu M, Ohko K, Yamane K & Tsuruo T (2000) Involvement of 14-3-3 proteins in nuclear localization of telomerase. *Embo J* **19**: 2652–2661
- Sekaric P, Shamanin VA, Luo J & Androphy EJ (2007) hAda3 regulates p14ARF-induced p53 acetylation and senescence. *Oncogene* **26**: 6261–8
- Sekimoto T, Fukumoto M & Yoneda Y (2004) 14-3-3 suppresses the nuclear localization of threonine 157-phosphorylated p27(Kip1). *Embo J* **23**: 1934–1942
- Selinka H-C, Giroglou T & Sapp M (2002) Analysis of the Infectious Entry Pathway of Human Papillomavirus Type 33 Pseudovirions. *Virology* **299**: 279–287
- Seong H-A & Ha H (2012) Murine protein serine-threonine kinase 38 activates p53 function through Ser15 phosphorylation. *The Journal of biological chemistry* **287**: 20797–810
- Shafti-Keramat S, Handisurya A, Krichuber E, Meneguzzi G, Slupetzky K & Kirnbauer R (2003) Different heparan sulfate proteoglycans serve as cellular receptors for human papillomaviruses. *Journal of virology* **77**: 13125–35
- Shai A, Brake T, Somoza C & Lambert PF (2007a) The human papillomavirus E6 oncogene dysregulates the cell cycle and contributes to cervical

- carcinogenesis through two independent activities. *Cancer Res* **67**: 1626–1635
- Shai A, Nguyen ML, Wagstaff J, Jiang Y-H & Lambert PF (2007b) HPV16 E6 confers p53-dependent and p53-independent phenotypes in the epidermis of mice deficient for E6AP. *Oncogene* **26**: 3321–8
- Shai A, Pitot HC & Lambert PF (2010) E6-associated protein is required for human papillomavirus type 16 E6 to cause cervical cancer in mice. *Cancer research* **70**: 5064–73
- Shamanin VA, Sekaric P & Androphy EJ (2008) hAda3 degradation by papillomavirus type 16 E6 correlates with abrogation of the p14ARF-p53 pathway and efficient immortalization of human mammary epithelial cells. *Journal of virology* **82**: 3912–20
- Shaulsky G, Goldfinger N, Ben-Ze'ev A & Rotter V (1990) Nuclear accumulation of p53 protein is mediated by several nuclear localization signals and plays a role in tumorigenesis. *Molecular and cellular biology* **10**: 6565–77
- Shen J, Person MD, Zhu J, Abbruzzese JL & Li D (2004) Protein expression profiles in pancreatic adenocarcinoma compared with normal pancreatic tissue and tissue affected by pancreatitis as detected by two-dimensional gel electrophoresis and mass spectrometry. *Cancer research* **64**: 9018–26
- Shen L & Turner JR (2005) Actin depolymerization disrupts tight junctions via caveolae-mediated endocytosis. *Molecular biology of the cell* **16**: 3919–36
- Shen YH, Godlewski J, Bronisz A, Zhu J, Comb MJ, Avruch J & Tzivion G (2003) Significance of 14-3-3 self-dimerization for phosphorylation-dependent target binding. *Molecular biology of the cell* **14**: 4721–33
- Sherman L & Schlegel R (1996) Serum- and calcium-induced differentiation of human keratinocytes is inhibited by the E6 oncoprotein of human papillomavirus type 16. *Journal of virology* **70**: 3269–79
- Shin I, Yakes FM, Rojo F, Shin N-Y, Bakin A V, Baselga J & Arteaga CL (2002) PKB/Akt mediates cell-cycle progression by phosphorylation of p27(Kip1) at threonine 157 and modulation of its cellular localization. *Nature medicine* **8**: 1145–52
- Shirasawa H, Tomita Y, Fuse A, Yamamoto T, Tanzawa H, Sekiya S, Takamizawa H & Simizu B (1989) Structure and expression of an integrated human papillomavirus type 16 genome amplified in a cervical carcinoma cell line. *The Journal of general virology* **70** ( Pt 7): 1913–9

- Short JD, Dere R, Houston KD, Cai S-L, Kim J, Bergeron JM, Shen J, Liang J, Bedford MT, Mills GB & Walker CL (2010) AMPK-mediated phosphorylation of murine p27 at T197 promotes binding of 14-3-3 proteins and increases p27 stability. *Molecular carcinogenesis* **49**: 429–39
- Siddhanti SR, Hartle JE & Quarles LD (1995) Forskolin inhibits protein kinase C-induced mitogen activated protein kinase activity in MC3T3-E1 osteoblasts. *Endocrinology* **136**: 4834–41
- Simonson SJS, Difilippantonio MJ & Lambert PF (2005) Two distinct activities contribute to human papillomavirus 16 E6's oncogenic potential. *Cancer research* **65**: 8266–73
- Skouloudaki K, Puetz M, Simons M, Courbard J-R, Boehlke C, Hartleben B, Engel C, Moeller MJ, Englert C, Bollig F, Schäfer T, Ramachandran H, Mlodzik M, Huber TB, Kuehn EW, Kim E, Kramer-Zucker A & Walz G (2009) Scribble participates in Hippo signaling and is required for normal zebrafish pronephros development. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 8579–84
- Slebos RJ, Habets GG, Evers SG, Mooi WJ & Rodenhuis S (1991) Allele-specific detection of K-ras oncogene expression in human non-small-cell lung carcinomas. *International journal of cancer. Journal international du cancer* **48**: 51–6
- Slee EA, Benassi B, Goldin R, Zhong S, Ratnayaka I, Blandino G & Lu X (2010) Phosphorylation of Ser312 contributes to tumor suppression by p53 in vivo. *Proceedings of the National Academy of Sciences of the United States of America* **107**: 19479–84
- Slingerland J & Pagano M (2000) Regulation of the cdk inhibitor p27 and its deregulation in cancer. *Journal of cellular physiology* **183**: 10–7
- Smith JL, Campos SK, Wandinger-Ness A & Ozbun MA (2008) Caveolin-1-dependent infectious entry of human papillomavirus type 31 in human keratinocytes proceeds to the endosomal pathway for pH-dependent uncoating. *Journal of virology* **82**: 9505–12
- Smith JS, Lindsay L, Hoots B, Keys J, Franceschi S, Winer R & Clifford GM (2007) Human papillomavirus type distribution in invasive cervical cancer and high-grade cervical lesions: a meta-analysis update. *International journal of cancer. Journal international du cancer* **121**: 621–32
- Smith-McCune K, Kalman D, Robbins C, Shivakumar S, Yuschenko L & Bishop JM (1999) Intracellular localization of human papillomavirus 16 E7 during transformation and preferential binding of E7 to the Rb family



- member p130. *Proceedings of the National Academy of Sciences of the United States of America* **96**: 6999–7004
- Smotkin D & Wettstein FO (1987) The major human papillomavirus protein in cervical cancers is a cytoplasmic phosphoprotein. *Journal of virology* **61**: 1686–9
- Snijders PJ, Steenbergen RD, Heideman DA & Meijer CJ (2006) HPV-mediated cervical carcinogenesis: concepts and clinical implications. *J Pathol* **208**: 152–164
- Son J, Park JW, Lambert PF & Chung S-H (2014) Requirement of estrogen receptor alpha DNA-binding domain for HPV oncogene-induced cervical carcinogenesis in mice. *Carcinogenesis* **35**: 489–96
- Song S, Gulliver GA & Lambert PF (1998) Human papillomavirus type 16 E6 and E7 oncogenes abrogate radiation-induced DNA damage responses in vivo through p53-dependent and p53-independent pathways. *Proc Natl Acad Sci U S A* **95**: 2290–2295
- Song S, Liem A, Miller JA & Lambert PF (2000) Human papillomavirus types 16 E6 and E7 contribute differently to carcinogenesis. *Virology* **267**: 141–150
- Song S, Pitot HC & Lambert PF (1999) The human papillomavirus type 16 E6 gene alone is sufficient to induce carcinomas in transgenic animals. *J Virol* **73**: 5887–5893
- Songyang Z, Fanning AS, Fu C, Xu J, Marfatia SM, Chishti AH, Crompton A, Chan AC, Anderson JM & Cantley LC (1997) Recognition of unique carboxyl-terminal motifs by distinct PDZ domains. *Science (New York, N.Y.)* **275**: 73–7
- Spadari S, Sala F & Pedrali-Noy G (1982) Aphidicolin: a specific inhibitor of nuclear DNA replication in eukaryotes. *Trends in Biochemical Sciences* **7**: 29–32
- Spangle JM & Münger K (2010) The human papillomavirus type 16 E6 oncoprotein activates mTORC1 signaling and increases protein synthesis. *Journal of virology* **84**: 9398–407
- Spanos WC, Geiger J, Anderson ME, Harris GF, Bossler AD, Smith RB, Klingelhutz AJ & Lee JH (2008a) Deletion of the PDZ motif of HPV16 E6 preventing immortalization and anchorage-independent growth in human tonsil epithelial cells. *Head Neck* **30**: 139–147

- Spanos WC, Hoover A, Harris GF, Wu S, Strand GL, Anderson ME, Klingelhutz AJ, Hendriks W, Bossler AD & Lee JH (2008b) The PDZ binding motif of human papillomavirus type 16 E6 induces PTPN13 loss, which allows anchorage-independent growth and synergizes with ras for invasive growth. *J Virol* **82**: 2493–2500
- Spoden G, Freitag K, Husmann M, Boller K, Sapp M, Lambert C & Florin L (2008) Clathrin- and caveolin-independent entry of human papillomavirus type 16--involvement of tetraspanin-enriched microdomains (TEMs). *PloS one* **3**: e3313
- Spurgeon ME, Chung S-H & Lambert PF (2014) Recurrence of cervical cancer in mice after selective estrogen receptor modulator therapy. *The American journal of pathology* **184**: 530–40
- St Clair S, Giono L, Varmeh-Ziaie S, Resnick-Silverman L, Liu W-J, Padi A, Dastidar J, DaCosta A, Mattia M & Manfredi JJ (2004) DNA damage-induced downregulation of Cdc25C is mediated by p53 via two independent mechanisms: one involves direct binding to the cdc25C promoter. *Molecular cell* **16**: 725–36
- Stacey SN, Jordan D, Williamson AJ, Brown M, Coote JH & Arrand JR (2000) Leaky scanning is the predominant mechanism for translation of human papillomavirus type 16 E7 oncoprotein from E6/E7 bicistronic mRNA. *J Virol* **74**: 7284–7297
- Stanley M (2008) Immunobiology of HPV and HPV vaccines. *Gynecologic oncology* **109**: S15–21
- Stanley MA, Browne HM, Appleby M & Minson AC (1989) Properties of a non-tumorigenic human cervical keratinocyte cell line. *International journal of cancer. Journal international du cancer* **43**: 672–6
- Stauffer Y, Raj K, Masternak K & Beard P (1998) Infectious human papillomavirus type 18 pseudovirions. *Journal of molecular biology* **283**: 529–36
- Stewart N, Hicks GG, Paraskevas F & Mowat M (1995) Evidence for a second cell cycle block at G2/M by p53. *Oncogene* **10**: 109–15
- Stokoe D, Macdonald SG, Cadwallader K, Symons M & Hancock JF (1994) Activation of Raf as a result of recruitment to the plasma membrane. *Science (New York, N.Y.)* **264**: 1463–7

- Storey A, Pim D, Murray A, Osborn K, Banks L & Crawford L (1988) Comparison of the in vitro transforming activities of human papillomavirus types. *The EMBO journal* **7**: 1815–20
- Straight SW, Hinkle PM, Jewers RJ & McCance DJ (1993) The E5 oncoprotein of human papillomavirus type 16 transforms fibroblasts and effects the downregulation of the epidermal growth factor receptor in keratinocytes. *Journal of virology* **67**: 4521–32
- Strati K & Lambert PF (2007) Role of Rb-dependent and Rb-independent functions of papillomavirus E7 oncogene in head and neck cancer. *Cancer Res* **67**: 11585–11593
- Su TT, Parry DH, Donahoe B, Chien CT, O'Farrell PH & Purdy A (2001) Cell cycle roles for two 14-3-3 proteins during *Drosophila* development. *Journal of cell science* **114**: 3445–54
- Subbaiah VK, Kranjec C, Thomas M & Banks L (2011) PDZ domains: the building blocks regulating tumorigenesis. *The Biochemical journal* **439**: 195–205
- Subbaiah VK, Massimi P, Boon SS, Myers MP, Sharek L, Garcia-Mata R & Banks L (2012) The invasive capacity of HPV transformed cells requires the hDlg-dependent enhancement of SGEF/RhoG activity. *PLoS pathogens* **8**: e1002543
- Sun L, Stoecklin G, Van Way S, Hinkovska-Galcheva V, Guo R-F, Anderson P & Shanley TP (2007) Tristetraprolin (TTP)-14-3-3 complex formation protects TTP from dephosphorylation by protein phosphatase 2a and stabilizes tumor necrosis factor- $\alpha$  mRNA. *The Journal of biological chemistry* **282**: 3766–77
- Sun L, Zhang G, Lei T, Huang C, Song T & Si L (2008) Two different HPV-11E6 fusion proteins trap p53 in the cytoplasm and induce apoptosis. *Cancer Biol Ther* **7**: 1909–1915
- Sun XY, Frazer I, Müller M, Gissmann L & Zhou J (1995) Sequences required for the nuclear targeting and accumulation of human papillomavirus type 6B L2 protein. *Virology* **213**: 321–7
- Suzuki H, Itoh F, Toyota M, Kikuchi T, Kakiuchi H & Imai K (2000) Inactivation of the 14-3-3 sigma gene is associated with 5' CpG island hypermethylation in human cancers. *Cancer research* **60**: 4353–7
- Swindle CS & Engler JA (1998) Association of the human papillomavirus type 11 E1 protein with histone H1. *Journal of virology* **72**: 1994–2001

- Tada K, Oka M, Tangoku A, Hayashi H, Oga A & Sasaki K (2000) Gains of 8q23-qter and 20q and loss of 11q22-qter in esophageal squamous cell carcinoma associated with lymph node metastasis. *Cancer* **88**: 268–73
- Takeda J, Adachi K, Halprin KM, Itami S, Levine V & Woodyard C (1983) Forskolin Activates Adenylate Cyclase Activity and Inhibits Mitosis in In Vitro in Pig Epidermis. *Journal of Investigative Dermatology* **81**: 236–240
- Takahara Y, Matsuda Y & Hara J (2000) Role of the beta isoform of 14-3-3 proteins in cellular proliferation and oncogenic transformation. *Carcinogenesis* **21**: 2073–7
- Talis AL, Huibregtse JM & Howley PM (1998) The Role of E6AP in the Regulation of p53 Protein Levels in Human Papillomavirus (HPV)-positive and HPV-negative Cells. *Journal of Biological Chemistry* **273**: 6439–6445
- Talora C, Sgroi DC, Crum CP & Dotto GP (2002a) Specific down-modulation of Notch1 signaling in cervical cancer cells is required for sustained HPV-E6/E7 expression and late steps of malignant transformation. *Genes Dev* **16**: 2252–2263
- Talora C, Sgroi DC, Crum CP & Dotto GP (2002b) Specific down-modulation of Notch1 signaling in cervical cancer cells is required for sustained HPV-E6/E7 expression and late steps of malignant transformation. *Genes & development* **16**: 2252–63
- Tan MJA, White EA, Sowa ME, Harper JW, Aster JC & Howley PM (2012) Cutaneous  $\beta$ -human papillomavirus E6 proteins bind Mastermind-like coactivators and repress Notch signaling. *Proceedings of the National Academy of Sciences of the United States of America* **109**: E1473–80
- Tang SJ, Suen TC, McInnes RR & Buchwald M (1998) Association of the TLX-2 homeodomain and 14-3-3beta signaling proteins. *The Journal of biological chemistry* **273**: 25356–63
- Tao M, Kruhlak M, Xia S, Androphy E & Zheng Z-M (2003) Signals That Dictate Nuclear Localization of Human Papillomavirus Type 16 Oncoprotein E6 in Living Cells. *Journal of Virology* **77**: 13232–13247
- Teufel DP, Bycroft M & Fersht AR (2009) Regulation by phosphorylation of the relative affinities of the N-terminal transactivation domains of p53 for p300 domains and Mdm2. *Oncogene* **28**: 2112–8
- Thomas JT, Hubert WG, Ruesch MN & Laimins LA (1999) Human papillomavirus type 31 oncoproteins E6 and E7 are required for the

- maintenance of episomes during the viral life cycle in normal human keratinocytes. *Proc Natl Acad Sci U S A* **96**: 8449–8454
- Thomas M & Banks L (1998) Inhibition of Bak-induced apoptosis by HPV-18 E6. *Oncogene* **17**: 2943–2954
- Thomas M & Banks L (1999a) Human papillomavirus (HPV) E6 interactions with Bak are conserved amongst E6 proteins from high and low risk HPV types. *J Gen Virol* **80** ( Pt 6): 1513–1517
- Thomas M & Banks L (1999b) Human papillomavirus (HPV) E6 interactions with Bak are conserved amongst E6 proteins from high and low risk HPV types. *The Journal of general virology* **80** ( Pt 6): 1513–7
- Thomas M, Laura R, Hepner K, Guccione E, Sawyers C, Lasky L & Banks L (2002) Oncogenic human papillomavirus E6 proteins target the MAGI-2 and MAGI-3 proteins for degradation. *Oncogene* **21**: 5088–96
- Thomas M, Massimi P & Banks L (1996a) HPV-18 E6 inhibits p53 DNA binding activity regardless of the oligomeric state of p53 or the exact p53 recognition sequence. *Oncogene* **13**: 471–480
- Thomas M, Massimi P, Navarro C, Borg JP & Banks L (2005) The hScrib/Dlg apico-basal control complex is differentially targeted by HPV-16 and HPV-18 E6 proteins. *Oncogene* **24**: 6222–6230
- Thomas M, Matlashewski G, Pim D & Banks L (1996b) Induction of apoptosis by p53 is independent of its oligomeric state and can be abolished by HPV-18 E6 through ubiquitin mediated degradation. *Oncogene* **13**: 265–273
- Thomas MC & Chiang CM (2005) E6 oncoprotein represses p53-dependent gene activation via inhibition of protein acetylation independently of inducing p53 degradation. *Molecular cell* **17**: 251–64
- Thorland EC, Myers SL, Gostout BS & Smith DI (2003) Common fragile sites are preferential targets for HPV16 integrations in cervical tumors. *Oncogene* **22**: 1225–37
- Thorson JA, Yu LW, Hsu AL, Shih NY, Graves PR, Tanner JW, Allen PM, Piwnicka-Worms H & Shaw AS (1998) 14-3-3 proteins are required for maintenance of Raf-1 phosphorylation and kinase activity. *Molecular and cellular biology* **18**: 5229–38
- Tomaíć V, Gardiol D, Massimi P, Ozbun M, Myers M & Banks L (2009a) Human and primate tumour viruses use PDZ binding as an evolutionarily

- conserved mechanism of targeting cell polarity regulators. *Oncogene* **28**: 1–8
- Tomać V, Pim D & Banks L (2009b) The stability of the human papillomavirus E6 oncoprotein is E6AP dependent. *Virology* **393**: 7–10
- Tommasino M & Crawford L (1995) Human papillomavirus E6 and E7: proteins which deregulate the cell cycle. *Bioessays* **17**: 509–518
- Tonikian R, Zhang Y, Sazinsky SL, Currell B, Yeh J-H, Reva B, Held HA, Appleton BA, Evangelista M, Wu Y, Xin X, Chan AC, Seshagiri S, Lasky LA, Sander C, Boone C, Bader GD & Sidhu SS (2008) A specificity map for the PDZ domain family. *PLoS biology* **6**: e239
- Töpffer S, Müller-Schiffmann A, Matentzoglou K, Scheffner M & Steger G (2007) Protein tyrosine phosphatase H1 is a target of the E6 oncoprotein of high-risk genital human papillomaviruses. *The Journal of general virology* **88**: 2956–65
- Traweger A, Fuchs R, Krizbai IA, Weiger TM, Bauer H-C & Bauer H (2003) The tight junction protein ZO-2 localizes to the nucleus and interacts with the heterogeneous nuclear ribonucleoprotein scaffold attachment factor-B. *The Journal of biological chemistry* **278**: 2692–700
- Tsunokawa Y, Takebe N, Kasamatsu T, Terada M & Sugimura T (1986) Transforming activity of human papillomavirus type 16 DNA sequence in a cervical cancer. *Proceedings of the National Academy of Sciences of the United States of America* **83**: 2200–3
- Tzivion G, Luo Z & Avruch J (1998) A dimeric 14-3-3 protein is an essential cofactor for Raf kinase activity. *Nature* **394**: 88–92
- Ubersax JA & Ferrell JE (2007) Mechanisms of specificity in protein phosphorylation. *Nature reviews. Molecular cell biology* **8**: 530–41
- Uchida D, Begum NM, Almofti A, Kawamata H, Yoshida H & Sato M (2004a) Frequent downregulation of 14-3-3 sigma protein and hypermethylation of 14-3-3 sigma gene in salivary gland adenoid cystic carcinoma. *Br J Cancer* **91**: 1131–1138
- Uchida S, Kuma A, Ohtsubo M, Shimura M, Hirata M, Nakagama H, Matsunaga T, Ishizaka Y & Yamashita K (2004b) Binding of 14-3-3beta but not 14-3-3sigma controls the cytoplasmic localization of CDC25B: binding site preferences of 14-3-3 subtypes and the subcellular localization of CDC25B. *Journal of cell science* **117**: 3011–20

- Uchida S, Ohtsubo M, Shimura M, Hirata M, Nakagama H, Matsunaga T, Yoshida M, Ishizaka Y & Yamashita K (2004c) Nuclear export signal in CDC25B. *Biochemical and biophysical research communications* **316**: 226–32
- van Der Hoeven PC, Van Der Wal JC, Ruurs P, Van Dijk MC & Van Blitterswijk J (2000) 14-3-3 isotypes facilitate coupling of protein kinase C-zeta to Raf-1: negative regulation by 14-3-3 phosphorylation. *The Biochemical journal* **345 Pt 2**: 297–306
- van Itallie CM & Anderson JM (1997) Occludin confers adhesiveness when expressed in fibroblasts. *Journal of cell science* **110 ( Pt 9)**: 1113–21
- Varshavsky A (1996) The N-end rule: functions, mysteries, uses. *Proceedings of the National Academy of Sciences of the United States of America* **93**: 12142–9
- Viglietto G, Motti ML, Bruni P, Melillo RM, D'Alessio A, Califano D, Vinci F, Chiappetta G, Tschlis P, Bellacosa A, Fusco A & Santoro M (2002) Cytoplasmic relocation and inhibition of the cyclin-dependent kinase inhibitor p27(Kip1) by PKB/Akt-mediated phosphorylation in breast cancer. *Nature medicine* **8**: 1136–44
- De Villiers E-M (2013) Cross-roads in the classification of papillomaviruses. *Virology* **445**: 2–10
- Vincenz C & Dixit VM (1996) 14-3-3 proteins associate with A20 in an isoform-specific manner and function both as chaperone and adapter molecules. *The Journal of biological chemistry* **271**: 20029–34
- Vinokurova S, Wentzensen N, Kraus I, Klaes R, Driesch C, Melsheimer P, Kisseliov F, Dürst M, Schneider A & von Knebel Doeberitz M (2008) Type-dependent integration frequency of human papillomavirus genomes in cervical lesions. *Cancer research* **68**: 307–13
- Vliet-Gregg PA, Hamilton JR & Katzenellenbogen RA (2013) NFX1-123 and human papillomavirus 16E6 increase Notch expression in keratinocytes. *Journal of virology* **87**: 13741–50
- Vousden KH, Doniger J, DiPaolo JA & Lowy DR (1988) The E7 open reading frame of human papillomavirus type 16 encodes a transforming gene. *Oncogene research* **3**: 167–75
- Walboomers JM, Jacobs M V, Manos MM, Bosch FX, Kummer JA, Shah K V, Snijders PJ, Peto J, Meijer CJ & Muñoz N (1999) Human papillomavirus

- is a necessary cause of invasive cervical cancer worldwide. *The Journal of pathology* **189**: 12–9
- Wallace NA & Galloway DA (2014) Manipulation of cellular DNA damage repair machinery facilitates propagation of human papillomaviruses. *Seminars in cancer biology*
- Wallace NA, Robinson K, Howie HL & Galloway DA (2012) HPV 5 and 8 E6 abrogate ATR activity resulting in increased persistence of UVB induced DNA damage. *PLoS pathogens* **8**: e1002807
- Wan PTC, Garnett MJ, Roe SM, Lee S, Niculescu-Duvaz D, Good VM, Jones CM, Marshall CJ, Springer CJ, Barford D & Marais R (2004) Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. *Cell* **116**: 855–67
- Wang B, Yang H, Liu Y-C, Jelinek T, Zhang L, Ruoslahti E & Fu H (1999) Isolation of High-Affinity Peptide Antagonists of 14-3-3 Proteins by Phage Display †. *Biochemistry* **38**: 12499–12504
- Wang H, Huang H, Li W, Jin X, Zeng J, Liu Y, Gu Y, Sun X, Wen G, Ding Y & Zhao L (2012) Nuclear localization of 14-3-3epsilon inversely correlates with poor long-term survival of patients with colorectal cancer. *Journal of surgical oncology* **106**: 224–31
- Wang Q, Griffin H, Southern S, Jackson D, Martin A, McIntosh P, Davy C, Masterson PJ, Walker PA, Laskey P, Omary MB & Doorbar J (2004) Functional analysis of the human papillomavirus type 16 E1=E4 protein provides a mechanism for in vivo and in vitro keratin filament reorganization. *Journal of virology* **78**: 821–33
- Wang Q, Kennedy A, Das P, McIntosh PB, Howell SA, Isaacson ER, Hinz SA, Davy C & Doorbar J (2009) Phosphorylation of the human papillomavirus type 16 E1–E4 protein at T57 by ERK triggers a structural change that enhances keratin binding and protein stability. *Journal of virology* **83**: 3668–83
- Watanabe S, Kanda T & Yoshiike K (1989) Human papillomavirus type 16 transformation of primary human embryonic fibroblasts requires expression of open reading frames E6 and E7. *J Virol* **63**: 965–969
- Watanabe S & Yoshiike K (1988) Transformation of rat 3Y1 cells by human papillomavirus type-18 DNA. *Int J Cancer* **41**: 896–900



- Waterman JL, Shenk JL & Halazonetis TD (1995) The dihedral symmetry of the p53 tetramerization domain mandates a conformational switch upon DNA binding. *The EMBO journal* **14**: 512–9
- Waterman MJ, Stavridi ES, Waterman JL & Halazonetis TD (1998) ATM-dependent activation of p53 involves dephosphorylation and association with 14-3-3 proteins. *Nature genetics* **19**: 175–8
- Wazer DE, Liu XL, Chu Q, Gao Q & Band V (1995) immortalization of distinct human mammary epithelial cell types by human papilloma virus 16 E6 or E7. *Proceedings of the National Academy of Sciences of the United States of America* **92**: 3687–91
- Werness BA, Levine AJ & Howley PM (1990) Association of human papillomavirus types 16 and 18 E6 proteins with p53. *Science* **248**: 76–79
- Wetherill LF, Holmes KK, Verow M, Müller M, Howell G, Harris M, Fishwick C, Stonehouse N, Foster R, Blair GE, Griffin S & Macdonald A (2012) High-risk human papillomavirus E5 oncoprotein displays channel-forming activity sensitive to small-molecule inhibitors. *Journal of virology* **86**: 5341–51
- White EA, Walther J, Javanbakht H & Howley PM (2014) Genus Beta HPV E6 Proteins Vary in their Effects on the Transactivation of p53 Target Genes. *Journal of virology* **88**: 8201–8212
- Wigler M, Pellicer A, Silverstein S, Axel R, Urlaub G & Chasin L (1979) DNA-mediated transfer of the adenine phosphoribosyltransferase locus into mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America* **76**: 1373–6
- Wilker EW, Grant RA, Artim SC & Yaffe MB (2005) A structural basis for 14-3-3sigma functional specificity. *J Biol Chem* **280**: 18891–18898
- Wilson VG, West M, Woytek K & Rangasamy D (2002) Papillomavirus E1 proteins: form, function, and features. *Virus genes* **24**: 275–90
- Winer RL, Kiviat NB, Hughes JP, Adam DE, Lee S-K, Kuypers JM & Koutsky LA (2005) Development and duration of human papillomavirus lesions, after initial infection. *The Journal of infectious diseases* **191**: 731–8
- Wood CE, Borgerink H, Register TC, Scott L & Cline JM (2004) Cervical and vaginal epithelial neoplasms in cynomolgus monkeys. *Veterinary pathology* **41**: 108–15

- Wood CE, Chen Z, Cline JM, Miller BE & Burk RD (2007) Characterization and experimental transmission of an oncogenic papillomavirus in female macaques. *Journal of virology* **81**: 6339–45
- Woodman CBJ, Collins SI & Young LS (2007) The natural history of cervical HPV infection: unresolved issues. *Nature reviews. Cancer* **7**: 11–22
- Woods DF & Bryant PJ (1991) The discs-large tumor suppressor gene of *Drosophila* encodes a guanylate kinase homolog localized at septate junctions. *Cell* **66**: 451–464
- Woods DF & Bryant PJ (1993) ZO-1, DlgA and PSD-95/SAP90: homologous proteins in tight, septate and synaptic cell junctions. *Mechanisms of development* **44**: 85–9
- Woods DF, Hough C, Peel D, Callaini G & Bryant PJ (1996) Dlg protein is required for junction structure, cell polarity, and proliferation control in *Drosophila* epithelia. *The Journal of cell biology* **134**: 1469–82
- Woodworth CD, Bowden PE, Doniger J, Pirisi L, Barnes W, Lancaster WD & DiPaolo JA (1988) Characterization of normal human exocervical epithelial cells immortalized in vitro by papillomavirus types 16 and 18 DNA. *Cancer research* **48**: 4620–8
- Woodworth CD, Doniger J & DiPaolo JA (1989) Immortalization of human foreskin keratinocytes by various human papillomavirus DNAs corresponds to their association with cervical carcinoma. *Journal of virology* **63**: 159–64
- Wright AA, Howitt BE, Myers AP, Dahlberg SE, Palescandolo E, Van Hummelen P, MacConaill LE, Shoni M, Wagle N, Jones RT, Quick CM, Laury A, Katz IT, Hahn WC, Matulonis UA & Hirsch MS (2013) Oncogenic mutations in cervical cancer: genomic differences between adenocarcinomas and squamous cell carcinomas of the cervix. *Cancer* **119**: 3776–83
- Wu J-W, Hu M, Chai J, Seoane J, Huse M, Li C, Rigotti DJ, Kyin S, Muir TW, Fairman R, Massagué J & Shi Y (2001) Crystal Structure of a Phosphorylated Smad2: Recognition of Phosphoserine by the MH2 Domain and Insights on Smad Function in TGF- $\beta$  Signaling. *Molecular Cell* **8**: 1277–1289
- Wu X, Hepner K, Castelino-Prabhu S, Do D, Kaye MB, Yuan XJ, Wood J, Ross C, Sawyers CL & Whang YE (2000a) Evidence for regulation of the PTEN tumor suppressor by a membrane-localized multi-PDZ domain

- containing scaffold protein MAGI-2. *Proceedings of the National Academy of Sciences of the United States of America* **97**: 4233–8
- Wu Y, Dowbenko D, Spencer S, Laura R, Lee J, Gu Q & Lasky LA (2000b) Interaction of the tumor suppressor PTEN/MMAC with a PDZ domain of MAGI3, a novel membrane-associated guanylate kinase. *The Journal of biological chemistry* **275**: 21477–85
- Xenophontos XP, Watson PA, Chua BH, Haneda T & Morgan HE (1989) Increased cyclic AMP content accelerates protein synthesis in rat heart. *Circulation research* **65**: 647–56
- Xiao B, Smerdon SJ, Jones DH, Dodson GG, Soneji Y, Aitken A & Gamblin SJ (1995) Structure of a 14-3-3 protein and implications for coordination of multiple signalling pathways. *Nature* **376**: 188–91
- Xiong Y, Kuppuswamy D, Li Y, Livanos EM, Hixon M, White A, Beach D & Tlsty TD (1996) Alteration of cell cycle kinase complexes in human papillomavirus E6- and E7-expressing fibroblasts precedes neoplastic transformation. *Journal of virology* **70**: 999–1008
- Xu M, Katzenellenbogen RA, Grandori C & Galloway DA (2010) NFX1 plays a role in human papillomavirus type 16 E6 activation of NFkappaB activity. *Journal of virology* **84**: 11461–9
- Xu M, Katzenellenbogen RA, Grandori C & Galloway DA (2013) An unbiased in vivo screen reveals multiple transcription factors that control HPV E6-regulated hTERT in keratinocytes. *Virology* **446**: 17–24
- Yaffe MB & Elia AE. (2001) Phosphoserine/threonine-binding domains. *Current Opinion in Cell Biology* **13**: 131–138
- Yaffe MB, Rittinger K, Volinia S, Caron PR, Aitken A, Leffers H, Gamblin SJ, Smerdon SJ & Cantley LC (1997) The Structural Basis for 14-3-3:Phosphopeptide Binding Specificity. *Cell* **91**: 961–971
- Yang H-Y, Wen Y-Y, Chen C-H, Lozano G & Lee M-H (2003) 14-3-3 sigma positively regulates p53 and suppresses tumor growth. *Molecular and cellular biology* **23**: 7096–107
- Yang J, Winkler K, Yoshida M & Kornbluth S (1999) Maintenance of G2 arrest in the Xenopus oocyte: a role for 14-3-3-mediated inhibition of Cdc25 nuclear import. *The EMBO journal* **18**: 2174–83
- Yang X, Lee WH, Sobott F, Papagrigoriou E, Robinson C V, Grossmann JG, Sundström M, Doyle DA & Elkins JM (2006) Structural basis for protein-

- protein interactions in the 14-3-3 protein family. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 17237–42
- Yao T-T, Dai Y-Z & Li S-Z (2008) [Expression and clinical significance of phosphatidylinositol 3-kinase and protein kinase B in cervical carcinoma]. *Ai zheng = Aizheng = Chinese journal of cancer* **27**: 525–30
- Yasumoto S, Burkhardt AL, Doniger J & DiPaolo JA (1986) Human papillomavirus type 16 DNA-induced malignant transformation of NIH 3T3 cells. *Journal of virology* **57**: 572–7
- Yatabe Y, Osada H, Tatematsu Y, Mitsudomi T & Takahashi T (2002) Decreased expression of 14-3-3sigma in neuroendocrine tumors is independent of origin and malignant potential. *Oncogene* **21**: 8310–9
- Yoo SH, Park BH, Choi J, Yoo J, Lee S-W, Kim Y-M & Kim K-R (2012) Papillary mucinous metaplasia of the endometrium as a possible precursor of endometrial mucinous adenocarcinoma. *Modern pathology: an official journal of the United States and Canadian Academy of Pathology, Inc* **25**: 1496–507
- Yoshihara K, Ikenouchi J, Izumi Y, Akashi M, Tsukita S & Furuse M (2011) Phosphorylation state regulates the localization of Scribble at adherens junctions and its association with E-cadherin-catenin complexes. *Experimental cell research* **317**: 413–22
- Yoshinouchi M, Yamada T, Kizaki M, Fen J, Koseki T, Ikeda Y, Nishihara T & Yamato K (2003) In vitro and in vivo growth suppression of human papillomavirus 16-positive cervical cancer cells by E6 siRNA. *Mol Ther* **8**: 762–768
- You J, Croyle JL, Nishimura A, Ozato K & Howley PM (2004) Interaction of the bovine papillomavirus E2 protein with Brd4 tethers the viral DNA to host mitotic chromosomes. *Cell* **117**: 349–60
- Yutsudo M, Okamoto Y & Hakura A (1988) Functional dissociation of transforming genes of human papillomavirus type 16. *Virology* **166**: 594–597
- Zanier K,ould M'hamed ould Sidi A, Boulade-Ladame C, Rybin V, Chappelle A, Atkinson A, Kieffer B & Travé G (2012) Solution structure analysis of the HPV16 E6 oncoprotein reveals a self-association mechanism required for E6-mediated degradation of p53. *Structure (London, England: 1993)* **20**: 604–17

- Zatsepina O, Braspenning J, Robberson D, Hajibagheri MA, Blight KJ, Ely S, Hibma M, Spitkovsky D, Trendelenburg M, Crawford L & Tommasino M (1997) The human papillomavirus type 16 E7 protein is associated with the nucleolus in mammalian and yeast cells. *Oncogene* **14**: 1137–45
- Zeng Y & Piwnica-Worms H (1999) DNA damage and replication checkpoints in fission yeast require nuclear exclusion of the Cdc25 phosphatase via 14-3-3 binding. *Molecular and cellular biology* **19**: 7410–9
- Zerfass-Thome K, Zwerschke W, Mannhardt B, Tindle R, Botz JW & Jansen-Dürr P (1996) Inactivation of the cdk inhibitor p27KIP1 by the human papillomavirus type 16 E7 oncoprotein. *Oncogene* **13**: 2323–30
- Zhan L, Rosenberg A, Bergami KC, Yu M, Xuan Z, Jaffe AB, Allred C & Muthuswamy SK (2008) Dereglulation of scribble promotes mammary tumorigenesis and reveals a role for cell polarity in carcinoma. *Cell* **135**: 865–78
- Zhang B, Chen W & Roman A (2006a) The E7 proteins of low- and high-risk human papillomaviruses share the ability to target the pRB family member p130 for degradation. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 437–42
- Zhang Y, Dasgupta J, Ma RZ, Banks L, Thomas M & Chen XS (2007) Structures of a human papillomavirus (HPV) E6 polypeptide bound to MAGUK proteins: mechanisms of targeting tumor suppressors by a high-risk HPV oncoprotein. *J Virol* **81**: 3618–3626
- Zhang Y, Yeh S, Appleton BA, Held HA, Kausalya PJ, Phua DCY, Wong WL, Lasky LA, Wiesmann C, Hunziker W & Sidhu SS (2006b) Convergent and divergent ligand specificity among PDZ domains of the LAP and zonula occludens (ZO) families. *The Journal of biological chemistry* **281**: 22299–311
- Zhao B, Li L, Tumaneng K, Wang C-Y & Guan K-L (2010) A coordinated phosphorylation by Lats and CK1 regulates YAP stability through SCF(beta-TRCP). *Genes & development* **24**: 72–85
- Zhao G-Y, Ding J-Y, Lu C-L, Lin Z-W & Guo J (2014) The overexpression of 14-3-3 $\zeta$  and Hsp27 promotes non-small cell lung cancer progression. *Cancer* **120**: 652–63
- Zheng L, Ding H, Lu Z, Li Y, Pan Y, Ning T & Ke Y (2008) E3 ubiquitin ligase E6AP-mediated TSC2 turnover in the presence and absence of HPV16 E6. *Genes to cells: devoted to molecular & cellular mechanisms* **13**: 285–94

## REFERENCES

- Zhou J, Stenzel DJ, Sun XY & Frazer IH (1993) Synthesis and assembly of infectious bovine papillomavirus particles in vitro. *The Journal of general virology* **74** ( Pt 4): 763–8
- Zimmermann H, Degenkolbe R, Bernard H-U & O'Connor MJ (1999) The Human Papillomavirus Type 16 E6 Oncoprotein Can Down-Regulate p53 Activity by Targeting the Transcriptional Coactivator CBP/p300. *J. Virol.* **73**: 6209–6219
- zur Hausen H (1991) Viruses in human cancers. *Science (New York, N.Y.)* **254**: 1167–73
- zur Hausen H (1996) Papillomavirus infections--a major cause of human cancers. *Biochimica et biophysica acta* **1288**: F55–78
- zur Hausen H & de Villiers EM (1994) Human papillomaviruses. *Annu Rev Microbiol* **48**: 427–447